THE UNIVERSITY OF NOTTINGHAM

SCHOOL OF BIOLOGY

MUCOSAL IMMUNITY TO

THE HOOKWORM *Ancylostoma ceylanicum*

by

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Dedication to:

This thesis is affectionately dedicated to my mother, father, my wife and daughters

ABSTRACT

The host-parasite relationship of the hookworm *Ancylostoma ceylanicum* was explored in a hamster model system, focusing on intestinal mucosal responses to infection.

Primary infection induced a rapid reduction in villous height culminating in excess of 75% reduction by day 35. Crypts of Lieberkuhn increased in depth achieving maximum depth by day 35. Mitotic figures in crypts and mast cells increased until day 28. Goblet cells increased continuously from background levels of 50 cell/mm^2 to exceed 300 cells/mm^2 by day 42. Paneth cell numbers declined in infected animals.

Termination of infection by anthelmintic restored background values of intestinal architecture and goblet cell numbers within 7days but mast cells took longer and Paneth cell numbers increased beyond values in naive controls. Mucosal changes are therefore dependent on the presence of worms, intensity of infection and change dramatically with time.

Mucosal changes were studied in hamsters experiencing secondary infections following anthelmintic abbreviation of the immunizing infection, superimposed challenge infection and trickle infections. The kinetics of the responses were compared to animals experiencing primary infections and naïve controls. Among the findings were:

1) continuous reduction in villous height and a marked increase in crypt depth from day 10 after challenge in abbreviated primary-challenged hamsters compared to little change in hamsters given superimposed challenge.

2) marked mast, goblet, and Paneth cell and eosinophil responses.

3) less intense mast cell responses in abbreviated primary-challenged compared to superimposed challenge animals

4) after a superimposed challenge poor goblet cell responses because levels were already high at the time of challenge, little change in Paneth cells but intense eosinophil responses

5) slower changes in mucosal architecture and mast cell responses in trickle-infected animals eventually exceeding those in primary infected animals.

6) less marked goblet and Paneth cell responses in trickle-infected groups but more intense, persistent increases in eosinophils

Cyclosporin A's (CsA) usefulness as an immunosuppressive therapy for blocking T cell control of immunity was explored. However, CsA turned out to have marked anthelmintic properties and reductions in worm burden confounded the interpretation of mucosal changes.

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ABBREVIATIONS

AWT	Adult worm transfer
CsA	cyclosporin A
DPX	distrene, Plasticizer, Xylene
EPG	eggs per gram
GALT	gut-associated lymphoid tissue
GI nematodes	Gastrointestinal Nematodes
IEL	interepithelial leucocytes
IL	Interleukin
INF-Y	interferon gamma
L	litre
L3	third stage larvae
L4	forth stage larvae
MALT	mucosal-associated lymphoid tissue
Min	minute
MT fixative	Mercuric chloride/Tannic acid fixative
PAS	periodic acid Schiff
PBS	Phosphate buffered saline
Pi	post infection
SC	subcutaneous
Th	T helper cell
TNF	tumour necrosis factor
VCU	villus crypt units

CHAPTER ONE

GENERAL INTRODUCTION

1.1– INTRODUCTION

Nematodes are invertebrate organisms classified in their own phylum, Nematoda, although there has been considerable debate about their precise relationship to other animals (Smyth, 1994). Opinions have varied as to whether the Nematoda constitute a phylum in their own right or whether they form a subdivision of the pseudocoelomate animals or other higher Taxa (Smyth, 1994). It is known that nematodes possess many characteristics that are intermediate between those possessed by flatworms and those possessed by animals that are more complex. Yet, their uniqueness is sufficient for biologists to regard their origin as a separate group of animals, rather than as an intermediate between acoelomates and eucoelomates (Figure 1.1).

Organisms classified as nematodes include a wide range of species that exploit different life styles and occupy many different niches. These include free-living species, plant and animal parasitic nematodes. Plant parasitic species are important pests in arable agriculture where they cause loss to crops that can be of economic significance. Animal parasitic species can cause infection in domestic animals such as cattle and sheep and represent a threat to livestock agriculture (Michel, 1976; Michel, 1982; Michel, 1985). Some animal parasitic nematodes have severe pathological effects on humans and they present a major health burden on a global scale (MacInnis, 1991) and therefore they are of considerable medical significance (Wakelin, 1996). Humans may become exposed to infection with helminth parasites in the early stages of their lives and remain infected until much later (Anderson and Medley, 1985; Trent, 1963). A key characteristic of helminth infections in man, such as those caused by *Trichinella*, hookworms, *Ascaris* and Filarial worms, is that they persist for a very long time causing chronic debilitation to the host's health (Rogers, 1986).

Gastrointestinal helminth infections continue to cause morbidity and mortality throughout the developing world (Who, 1981). These parasites, such as *Ascaris*, muscle worms and hookworms occupy a variety of niches within the host gut and rejection is often accompanied by gross intestinal inflammation (Finkelmen *et al.*, 1997; Urban *et al.*, 1995). Hookworm disease, which is widely

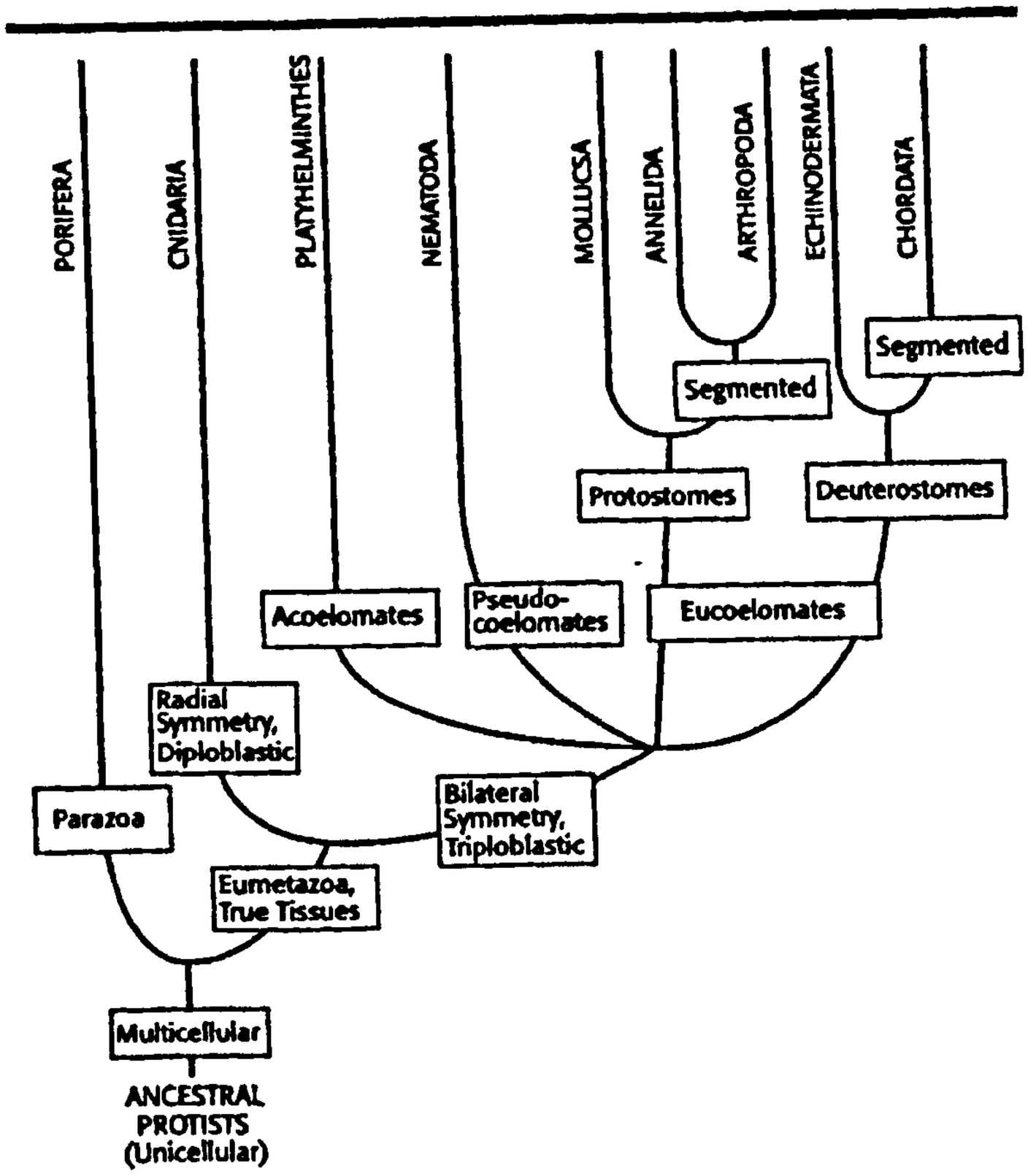


Figure 1.1 – Phylogeny showing probable relationships among the major animal phyla (Nematode-Pseudocoelomates). (Hopkins and Smith, 1997).

spread among humans living in the equatorial tropical and subtropical areas, is still one of the major diseases to affect mankind. It has also been suggested that hookworm infections are the most widespread as well as clinically one of the most important diseases caused by soil-transmitted helminths in some developing countries (Singh and Cox-Singh, 2001). Vast areas of Africa, the Middle East and Asia are endemic for hookworm infection as these areas provide the requirements for hookworm life cycles to be completed, such as warmth and moisture. However, because of marked contrast in the earth's surface, rainfall, vegetation and other climatic aspects (Schad and Warren, 1990) regions where hookworm exist vary among continent. Hookworm disease, combined with other parasitic infection, such as malaria and malnutrition causes misery to the human population throughout their endemic areas and where several infections co-occur life can be precarious and untenable.

Hookworms are parasites of mammals that infect a range of species. Different animal hosts were investigated in the early days of hookworm research including humans, carnivores and ungulates. Looss (1911) was among the first who investigated and described the developmental biology, life cycle and migration of *A. duodenale* in dogs and his investigations remain useful until today. Another series of studies using *A. caninum* infections in dogs were carried out by Foster and Cort, (1931) to demonstrate the acquired resistance to hookworm infections. There was no suitable rodent model of adult hookworm infection, and therefore workers have concentrated on dogs (Carroll and Grove, 1984; Carroll and Grove, 1985b) and cats (Yoshida *et al.*, 1968).

In 1972a, Ray and his co-workers studied parasitological aspects of the *A.ceylanicum* (dog strain) adapted for passage in hamsters. Six years later, Ray *et al.* (1978) showed that the *A. ceylanicum* model was useful for evaluation of various anthelmintic drugs and it has been used often for chemotherapeutic trials since then. However, many aspects of the host-parasite relationship had not been covered by the early research on hookworms and these formed the subject of later research (Garside and Behnke, 1989; Norozian-Amiri and Behnke, 1993). Much of this earlier work concentrated on the details of the host-parasite relationship, including development of the worm in hamsters,

larval survival and longevity of worm (Norozian-Amiri, 1993). Later immunological aspects were explored including antibody responses and mucosal changes (Garside *et al.*, 2000). Although, *A.ceylanicum* does not occur frequently in humans because it is primarily a parasite of carnivores, it differs from other hookworm species in being able to develop in a relatively larger range of hosts (Behnke, 1990; Ray *et al.*, 1972a; Ray *et al.*, 1972b). Thus, not only can this species infect dogs but Indian workers developed a strain that proved to be infective to hamsters. This strain, the hamster-adapted strain of *A. ceylanicum*, has been maintained in hamsters since the 1970s in India but also in Nottingham and other laboratories throughout the world. Although hamsters are not ideal hosts (since they are not as widely used as mice and because immunological reagents for hamster research are still limited), their relative cheapness in contrast to dogs and simpler husbandry facilitated larger experiments providing quantitative data for analysis. (Carroll and Grove, 1984).

1.2 – CLASSIFICATION

1.2.1 – *Ancylostoma ceylanicum*

Classification of the nematodes is complex and even now there is little agreement on the precise relationship of various orders, families and genera to each other (Loose, 1905, 1911; Smyth, 1994). However, hookworms belong to the family Ancylostomatidae and the full classification is generally given as the following:

- Phylum Nematode
- Class Phasmidea
- Order Eunematode
- Super Family Ancylostomatoidea (Strongyloidea)
- Family Ancylostomidae

- **A- Sub Family** Ancylostomatinae
- Genus Ancylostoma
- Species:

    1. *A. duodenale* (Man)
    2. *A. ceylanicum* (Man, dog and cat)
    3. *A. braziliense* (dog and cat)
    4. *A. tubaeforme* (cat)
    5. *A. caninum* (dog)

- **B- Sub family** Bunostominae (Necatorinae)
- Species:

    1. *N. americanus* (Man)
    2. *B. phlebotomum* (Cow)
    3. *B. trigonocephalum* (Sheep)
    4. *G. pachyselis* (Sheep)

As can be seen from the Figure 1.2, which has been taken from Lichtenfels, J.R (1980), the two human genera, *Ancylostoma* and *Necator*, are classified in different subfamilies, Ancylostomatinae and Bunostominae.

1.2.2 – *Trichinella spiralis*

Another parasite included in this study is the muscle worm *Trichinella spiralis*. Its relationship with the host is well known and covered by many scientists (Bell and Liu, 1988; Wakelin, 1996). *Trichinella spiralis* follows a different classification to the hookworm. This species is included under a different super family, which is the Trichuroidea. Both parasites used in the work described in this thesis, the *Ancylostoma* and *Trichinella*, are intestinal

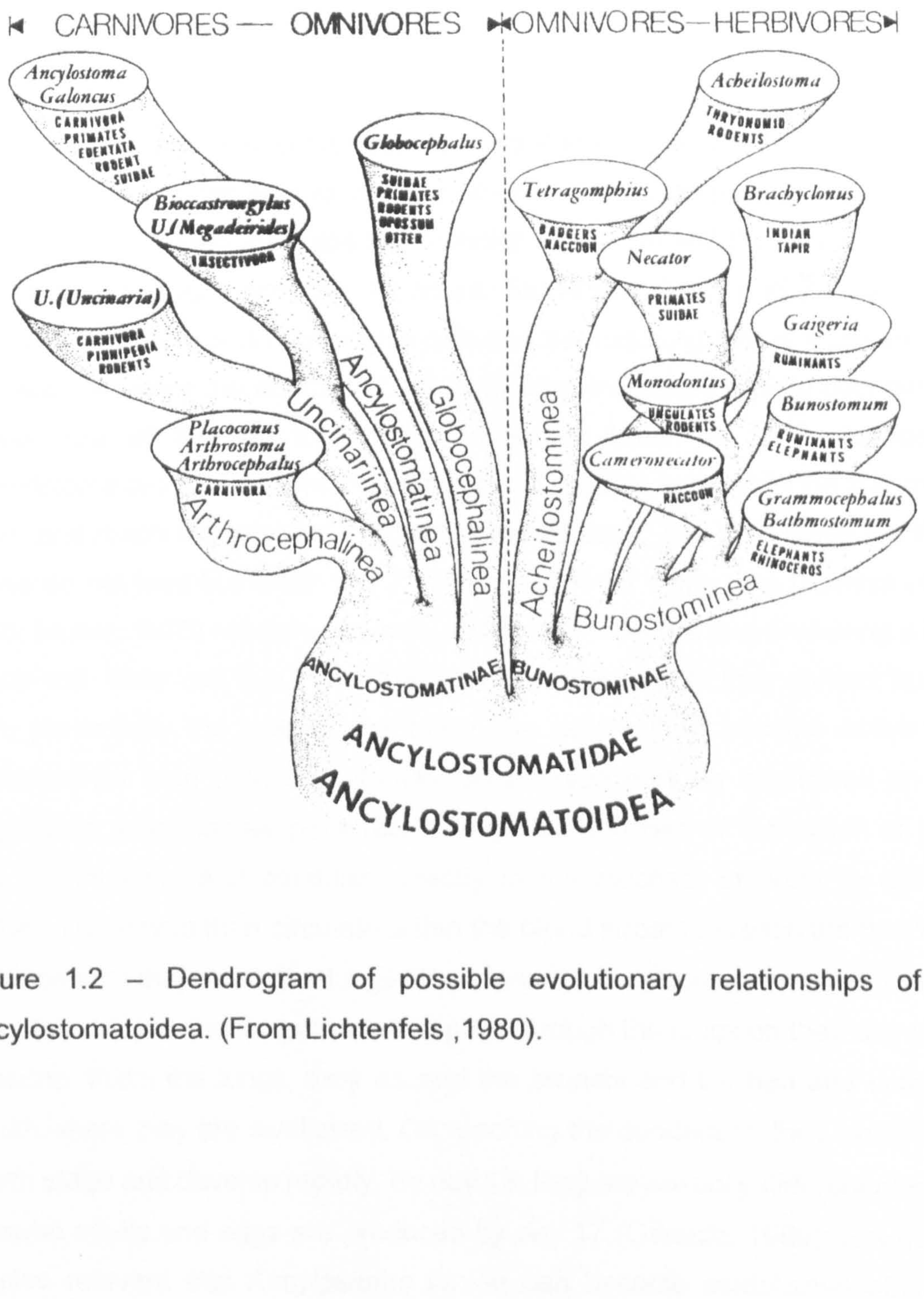


Figure 1.2 – Dendrogram of possible evolutionary relationships of the Ancylostomatoidea. (From Lichtenfels ,1980).

helminths and were chosen to compare their effects on their hosts as they are of interest to animal and human parasitologists.

1.3 – LIFE CYCLE

1.3.1- *Ancylostoma ceylanicum*

The life cycles of *A. ceylanicum* is illustrated in figure 1.3.1. It begins when fertilised adult females lay oval and thin-shelled eggs to be passed in the faeces (Muller, 1975). When the eggs reach moist and worm soil the larvae develop and hatch out in approximately 24 hours. Studies by Smith and Schad (1989) indicated that there is a relationship between the hatching rate of eggs and the temperature where the number of hatching eggs increased rapidly over time at temperature of 35 and took much longer to hatch at 15°C. Larvae of *Ancylostoma ceylanicum* feed on bacteria and organic remains in the soil, moult twice, and reach the infective third stage in five days. The third stage filariform larvae do not feed but retain the 2nd stage cuticle for some time (Komiya *et al.*, 1965; Muller, 1975) requiring warmth, shade and moisture and preferring a light sandy soil. They can live in the soil for a few weeks until they contact human skin, particularly the web of the toes. The larvae then become active and penetrate the skin (although *Ancylostoma* larvae may be swallowed on raw vegetables, when larvae penetrate through the mucosa of the mouth or pass into the intestine and establish directly in the mucosa) in order to develop further. The larvae then circulate within the blood stream to reach the heart and continue until they reach the lungs. The larvae of *Ancylostoma* species probably do not develop in the lungs but rather pass through the lungs on their way to the intestine. From the lungs, they ascend the bronchi and trachea and enter the mouth where they are swallowed. On reaching the duodenum, they moult to the fourth stage and develop rapidly. By day 13, they are sexually differentiated and became adults and eggs are produced by day 17 (Garside, 1989). However, it is also relevant that *Ancylostoma* larvae can become established after oral

ingestion. In this case, they do not migrate through the tissues and organs of the host, but develop into adults in the gut.

1.3.1.1- Life span of adult hookworm

Hookworms of man have often been considered to be long-lived nematode parasites. Evidence for this longevity was provided by self infection with *Necator*, which was shown to survive up to 17 years (Palmer, 1955) and 18 years (Beaver, 1988) in the absence of re-infection. This was based on the belief that the parasite entered the host and developed within a normal pre-patent period (days 32-35), and that both *N.americanus* and *A. duodenale* possibly have similar longevity. In contrast to *N. americanus*, *A. duodenale* is known to be able to adopt a hypobiotic state at an early stage, often referred to as arrested development and this phenomenon is believed to be triggered by seasonal and climatic factors (Schad *et al.*, 1973). This means that the worms can survive as larvae for a long time. An attempt to estimate the life-span of *N.americanus* and *A. duodenale* was carried out by (Nawalinski *et al.*, 1978) on Bengali children. He found that the average life span for *N. americanus* was about 3-4 years and that of *A. duodenale* about 1 year under the conditions of re-infection found in the study area. Other scientists showed that the life-span of *A. duodenale* was about one year to 18 months (Saint-Martin and Dussault, 1975), 4-6 years (Boycott, 1911), hookworm of both species 8 years (Chandler, 1926; Chandler, 1935) and varied seasonally. Carroll and Grove (1984) estimated that *A.ceylanicum* survived in dogs for more than 36 weeks by monitoring the faecal egg counts. This is very similar to that seen in humans infected with *A. ceylanicum*, where as Visen, *et al.* (1984) concluded that this species only survives between 27-48 days in hamsters. In contrast, it was found by Garside and Behnke (1989) that *A.ceylanicum* burdens remain stable for at least 7-10 weeks in hamsters. However, the survival of some hookworm species such as *N.americanus* is dependant on initial infection intensity (Rose and Behnke, 1990).

1.3.2- *Trichinella spiralis*

The sexes of *Trichinella spiralis* are dissimilar and can be easily distinguished. Males are smaller and more slender than females (Smyth, 1994). Infection in animals and man is acquired by the host eating raw or undercooked flesh containing encapsulated larvae. Cyst larvae may remain viable with no further development for several years. Larvae are released during digestion in the hosts' stomach and penetrate the mucosa and carry on into muscles, which is where they complete their developmental phase. Shanta and Meerovitch (1967) suggested that larvae commence penetrating the muscles on day 6. By day 17 they begin to coil, then the formation of the cyst wall can be detected by the day 21. When the infected muscles are eaten by the next host the larvae become activated, grow quickly, moult 4 times and reach sexually maturation rapidly in around 30 hours (Muller and Baker, 1990)(Fig 1.3.2).

1.4 – TYPES OF INFECTION

1.4.1 – Primary infection

When a naïve host is exposed experimentally to a large number of parasitic larvae at one single point in time, this is referred to as a single pulse primary infection and if this a first infection with the species of parasite, it is considered to be a primarily infection with that parasite. Subsequent infections are termed secondary and are often referred to as challenge infections. Frequently repeated, low doses infection, are known as trickle infection.

The duration of a single pulse primary infection can differ markedly between species of parasites. Some may be very prolonged and these are called chronic infection. Others may be brief, self limiting and acute in nature. Many nematodes of medical and economic importance are long lasting and thus cause chronic infections (reviewed by Behnke, 1987) as short-lived, self limiting

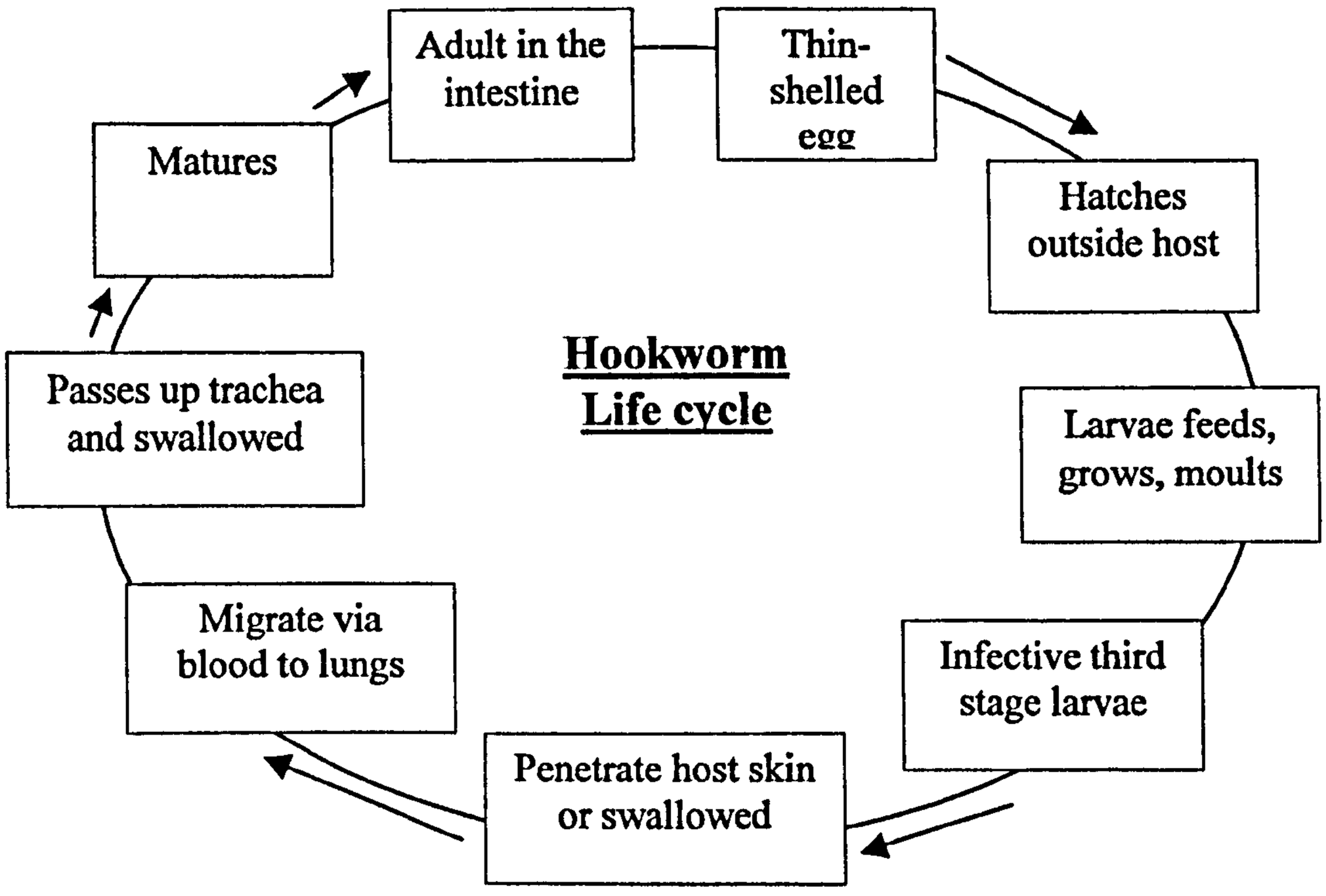


Figure. 1.3.1 – Life cycle of hookworms (e.g. *Ancylostoma ceylanicum*).

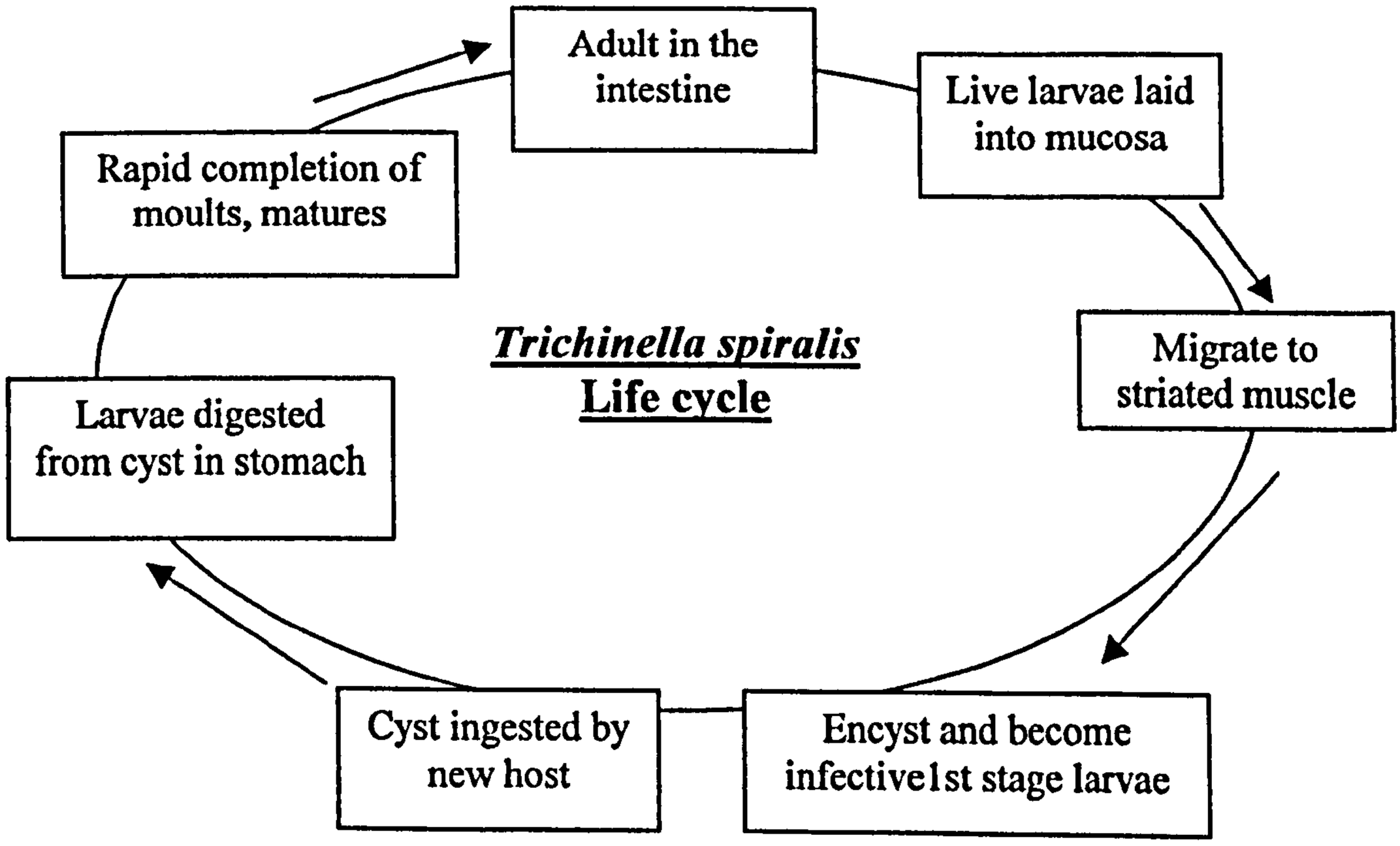


Figure 1.3.2 – Life cycle of *Trichinella spiralis*

infections are more characteristic of *T. spiralis*, *N. brasiliensis*, *T. muris* and *S. ratti*. These are expelled by mice and rats within 5 weeks of infection (Miller, 1984).

The mechanisms that facilitate prolonged nematode infection in vertebrates are still not fully understood. Behnke (1987b) and later Maizels, *et al.*, (1993) reviewed the potential of complex immunomodulatory strategies by parasites to down regulate the protective host response and a variety of mechanisms were thought to exist. Others also explored the wide range of mechanisms, which nematodes employ to evade the host protective immune responses, facilitating chronic worm survival. Despite the chronic survival of worms and the failure of the host to eliminate the adult parasites, some regulatory mechanisms are thought to exist. One of these is density-dependent regulation of fecundity and growth (Norozian-Amiri and Behnke, 1993).

A. ceylanicum together with the human hookworm *N. americanus* are only capable of long-term survival in human and hamster hosts (Behnke and Pritchard, 1987; Carroll and Grove, 1986; Garside and Behnke, 1989; Menon and Bhopale, 1985; Ray and Bhopale, 1972; Rose and Behnke, 1990; Yoshida *et al.*, 1971). Although high intensity primary infections in hamsters may be shorter in duration, complete expulsion of worms does not occur in this species following primary exposure (Rose and Behnke, 1990). Moreover, density-dependant constrains on growth were not evident during the larval phase of infection when the range was between 10-1000 larvae (Norozian-Amiri and Behnke, 1993). However, an experiment in which the range was extended to 1500 L3s gave a significant negative correlation between the magnitude of the infecting dose and percentage establishment.

1.4.2 –Multiple infection

The administration of multiple regular infections is called a trickle infection regime and this repeated infection regime is aimed at mimicking natural field conditions under which animals are continuously exposed to infection. Grazing ruminants take in large quantities of fresh infective larvae daily when feeding on

contaminated pasture, orders of magnitude higher than would be realistically expected in hookworm infections. The turnover of *Ostertagia* species by loss of adult worms and replacement with new larvae was a characteristic feature of this type of infection in cattle (Michel, 1970; Michel, 1976). *H. contortus* and *H. polygyrus,* however, show a different pattern, indicating that their survival strategies share common attributes (Brailsford and Behnke, 1992b).

The continuous intake of larvae in sheep subjected to trickle infection with *Trichostrongylus colubriformis* resulted in accumulation of worm burdens for three to six weeks until resistance developed to incoming larvae and the increase in adult worms was slowed down (Douch *et al.*, 1986). However, when administered larvae were abolished completely, stability in worm burdens was noted for varying period of time before they also were ultimately expelled (Donald and Waller, 1982; Jackson *et al.*, 1983). It was also found that low level infections showed stable unaltered worm burden until week 15, but when heavier larval challenges were given, worm burdens declined rapidly over a shorter period of time. Similarly, resistant mouse strains given trickle infection with *H. polygyrus* showed accumulation of worm burdens until a point when stability followed (Slater and Keymer, 1986).

Trickle infections with a hamster-adapted strain of *A. ceylanicum* revealed that worm burdens were regulated at a very low level, and this was found to be independent of the size of the infective dose (Brailsford and Behnke, 1992b). Again, it is likely that there is some turnover of adult worms during this type of infection. This regulation is immunologically mediated and both worm burden and fecundity were increased in hamsters that were immunosuppressed by treatment with cortisone (a powerful anti inflammatory steroid). The resistance observed during trickle infections was incomplete and some worms were found to survive continuously in repeatedly infected hamsters, for over 10 weeks. Although the infection appeared to be regulated by the hamsters, some worms were capable of surviving despite the host immune effectors (Brailsford and Behnke, 1992).

1.5 – IMMUNITY

1.5.1 – Innate immunity

Pathogens or foreign materials that successfully enter the body for the first time, will encounter barriers. These include innate and adaptive acquired immunity. Generally, it is known that innate immunity in the case of most pathogenic invaders is activated immediately following infection through recognition of presented epitopes associated with micro-organism, such as glycolipids. Similarly, antigens released from nematode bodies or secreted during invasion activate effector mechanisms inside the host body. This trigger of immune responses by foreigner invaders will lead to release of antimicrobial peptides, phagocytes and activation of the alternative complement pathway (Kamal, 2001; Kopp and Medzhitov, 1999). Those antimicrobial peptides have been demonstrated to be responsible of killing various bacteria, fungi, parasitic protozoa (reviewed in Mahida *et al.*, 1997) and nematode (Pillai, A. *et al.* 2003). Host specificity to particular parasite may indicate the existence of innate mechanism or barrier to further development that can be based on physical elements such as an inability to penetrate the skin or migrate further. Such a narrow host range among hookworms can be seen in the case of *N. americanus,* which is primarily a parasite of humans. However, it is also known to develop in other animals and experimentally limited infections can be achieved in infant rabbits, neonatal hamsters and young adult hamsters when corticosteroids are administered (Rajasekariah *et al.*, 1987). The human hookworm *N. americanus* develops incompletely in albino mice (Ray *et al.*, 1972a) which might be related to some genetic background of the host (Wells and Behnke, 1988), but is probably determined by innate resistance.

The rapid decline in hookworm recovery at the larval stages in the intestines of mice suggests that hookworms are unable to feed normally and do not survive in the intestines of mice or that rapid immune responses occur to eliminate the worms. Hosts respond to invasion by hookworms differently.

Studies in mice infected with different species of hookworms showed that larval stages could be recovered alive 6 months after first infection from the muscles in the case of mice infected with *A. duodenale* (Sasada, 1935b), 12 months in case of *A.caninum* infection (Nichols, 1956), 10 and 18 months in case of *A. tubaeforme* and *A. brasiliensis* respectively (Norris, 1971). This suggests that different species of hookworm have varying levels of success in surviving in abnormal hosts. Some feed and grow better than others in particular hosts but in each case, growth and development is optimal only in the preferred definitive host. Studies by Ray, *et al.* (1975) and Carroll, *et al.* (1983) showed that *A. ceylanicum* differ from *A.caninum* in completing their development in dogs, cats, man and experimentally infected hamsters but not in guinea pigs, rats or ferrets. Some Australian native rodents and immunocompromised outbred and inbred mice are not suitable hosts for *A.caninum* to complete its development; however, the species develops well only in dogs (Warner, 1998).

1.5.2 – The adaptive immune response and the specific role of T cells

Adaptive immunity is specific for the inducing agent and is marked by an enhanced response on repeated encounters with that agent. Thus, the key features of the adaptive immune response are memory and specificity and the ability to convert an initial encounter with antigen into a major response that activates a variety of effector cells (Behnke, 1991).

Immune responses to invading pathogens begin with the recognition of foreigner molecules and the subsequent induction of lymphocytes that have specificity for foreigner epitopes on surface of molecules. Central to the efficient induction and subsequent control of the immune response are T lymphocytes. These exert their controlling influence on other components of the immune response through cytokines.

Cytokines are small secretory glycoproteins, which play a regulatory role in the immune system. The role of these cytokines in parasitic infections has been elucidated by administering cytokines to infected animals, or eliminating them with monoclonal antibodies (Else and Finkelman, 1999; Finkelmen *et al.*,

1997) and the use of knock out animals in which specific genes have been inactivated or transgenic animals in which the genes have been up-regulated. There are many different types of cytokines and these include interleukins, interferons and colony-stimulation factors (Else and Finkelman, 1999).

It is known that cytokines not only act on various cells, including effector cells such as eosinophils, macrophages and mast cells, activating genes that subsequently alter cellular activity. Among the consequence of appropriate stimulation by cytokines can be up regulation of gene expression, up regulation of receptor expression and enhancement of phagocytic activity. Cytokines also act as important growth factors during the maturation of cellular response. Examples include the accumulation of macrophages and eosinophils in the granulomas in the liver during Schistosomiasis, eosinophilia and mast cell accumulation in the gut mucosa during worm infections of the gastrointestinal tract, both of which are controlled by appropriate release of relevant cytokines (eosinophilia –IL-5, mastocytosis IL-3, IL-4, IL-9 and IL-10) (Roitt *et al.*, 1998).

The type of T cell responsible for controlling an infection varies with the parasite and the stage of infection. Conventionally, two different types of T cells are recognised, differing in key surface molecules known as CD4 and CD8. In combination with the T cell receptor $CD4^+$ T cells interact with MHC class II antigen presenting molecules and this leads to a T helper response for the antigen presented by the macrophage. $CD8^+$ T cells interact with MHC class I antigen presenting molecules and on activation by specific antigen become cytotoxic (Cox and Liew, 1992; Roitt *et al.*, 1998). The $CD4^+$ T cells act in different ways in different infections. They are divided into three subsets, Th0, Th1 and Th2 (Cox and Liew, 1992; Else and Grencis, 1991a; Mosmann *et al.*, 1986), Th0 cell being generally considered to be precursors of Th1 and Th2 cells. The later are defined by the set of cytokines they secrete (Else and Finkelman, 1998). These cytokines are IL-2, lymphotoxin (TNF-ß) and IFN γ (secreted by Th1) and IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 (secreted by Th2). Th2 cytokines are required to drive the intestinal inflammatory response (Else and Finkelman, 1998). Both cells produce IL-3, granulocyte-macrophage colony stimulation factor (GM-CSF) and TNF-α (Kamal, 2001).

Several studies with different nematode species have showed that protective immunity against GI nematode is T cell-dependant. DiNetta *et al.*, (1972 and Larsh *et al.*, (1972) reported that treatment of mice with anti-thymocyte serum resulted in delayed expulsion of adult *T. spiralis* worms. Works with other nematode species, such as *N. brasiliensis*, have corroborate those finding (McKay *et al.*, 1995). Adoptive transfer experiments and *in vivo* depletion of $CD4^+$ T cells by cytotoxic anti-CD4 monoclonal antibodies (mAb) treatment have demonstrated that $CD4^+$ helper T cells play a role in the T cell-dependant protective immunity against GI nematode (Else and Finkelman, 1998; Finkelmen *et al.*, 1997). Also, studies in rodent infected with nematode, such as mice infected with *T. spiralis* and rats infected with *N. brasiliensis*, revealed that it was the $CD4^+$ T cells which are responsible of protective immunity which by the end leads to worms being expelled from the intestine (Grencis *et al.*, 1985; Wang *et al.*, 1990).

Both Th1 and Th2 responses are important in helminth infections (Roitt *et al.*, 1998). Among the hallmarks of the immune response to worm infections are IgE and eosinophil responses and these are dependent on Th2 cells. IL-5 is required for eosinophilia and IgA antibody responses while IL-4 is required for IgE antibody responses (Zhou *et al.*, 1996) and to regulate the enteropathy in some intestinal nematode infections (e.g. *T. spiralis*) (Lawrence *et al.*, 1998). Th2 cells are also responsible for the proliferation of B cells and humoral immunity (Kamal, 2001). However, in the context of the present work, Th2 cells drive intestinal inflammatory response. Immunological resistance to worm infection is associated with IgE, IgG, IgA antibody responses, mastocytosis and eosinophilia and all of these responses are dependent on cytokines secreted by Th2 lymphocytes. Goblet cell hyperplasia is also thought to be partially driven by IL-13 which is a Th2 cytokine and the marked changes in the intestinal architecture are dependent on TNF possibly from mast cells (Roitt *et al.*, 1998).

1.5.3 – Acquired immunity

Hookworm infections are generally long-lived and in humans do not induce strong protective responses (Behnke, 1987b). There is little evidence of protection on reinfection (Norozian-Amiri, 1993; Ottesen, 1990; Quinnell *et al.*, 1993). However, in canine models and in hamsters, immunity has been shown to be marked (Garside *et al.*, 1990; Gupta and Katiyar, 1985; Menon and Bhopale, 1985a). Nevertheless, even in these models, immunity is relatively weak compared to that known to exist in rodents infected with *T. spiralis* (Behnke *et al.*, 1994) and *N. brasiliensis.* In mice exposed once or repeatedly to oral larval challenge with *A. caninum*, resistance is generated and expressed as a reduction in the number of worms successfully passing through the intestinal barrier. It was also found that only a few larvae of *A. duodenale* migrate to the body organs hence recovery of arrested larvae from the tissue is significantly reduced (Sasada, 1935) However, it is not known yet whether larvae are prevented from penetrating or whether penetration was successful but followed by entrapment within the mucosa.

A. ceylanicum infection in dogs was studied extensively by Carroll and Grove (1985 and 1986). They studied acquired resistance to *A. ceylanicum* in dogs exposed to a primary infection of 2000 larvae 28 weeks before challenge. Residual worms were eliminated by chemotherapy 4 weeks before administration of the challenge infection with 1800 larvae. Reinfected dogs showed a marked reduction in fecal egg counts relative to control dogs and by week 6, a reduction of 85% in worm burden was recorded (Carroll and Grove, 1985). Moreover, dogs infected with a large number of infective larvae during an existing hookworm infection showed the capacity to reduce the number of adult worms that develop in the gut (Carroll and Grove, 1986). Cellular, serological and haematological changes were measured but the only noteworthy increase was in serum anti-hookworm IgA antibodies 2-3 weeks post challenge. Prior exposure may also facilitate a reduction in numbers of worms establishing themselves in the gut or earlier expulsion or both. The stimulation of resistance in hamsters by vaccination with UV-irradiated *A. ceylanicum* larvae was carried

out by Menon and Bhopale (1985a). They found a 95% reduction in adult worm burden in immunized animals compared with control hamsters.

Adoptive and passive transfer studies based respectively on injection of lymphocytes and serum from immune animals indicate that immune responses play a role in acquired resistance. When either serum or serum and lymph node cells were transferred to naïve dogs, they were protected and the establishment of challenge infection by *A. caninum* was reduced compared with controls (Miller, 1967)

1.6 – SMALL INTESTINE (ANATOMY AND FUNCTION)

The small intestine is the organ in which major enzymatic hydrolysis of the macromolecules in food occurs after the initial digestion carried out in the mouth and stomach (Grollman, 1974). It is critically important to the host as the principal site for the uptake of the breakdown of food. It is known that after mastication and digestion, food is broken down into its elementary molecules (Stanier and Forsling, 1990). Some of the larger molecules are further digested into amino acids, polypeptides, sugars and fats and these small molecules are internalised by diffusion and active transport systems associated with the enterocytes that line the mucosa (Stanier and Forsling, 1990). The breakdown products of digestion are then carried to the liver and other organs through the blood or initially via the lymphatic system (Grollman, 1974).

The organisation of the different layers that comprise the small intestine is illustrated in Figure 1.4 - A (Burkitt *et al.*, 1993) and B (Brooks and Paynton-Brooks, 1980). As can be seen the lumen is usually a collapsed space on the inside that swells as food expands the gut, driven along it length by peristalsis (Stanier and Forsling, 1990). The lumen of the gut is bordered by enterocytes that comprise the outer cell barrier of the mucosa. The internal lining is a thin overlaying mucosal membrane, which forms a protective barrier and protects the underlying tissues from pathogenic organisms via complex defence

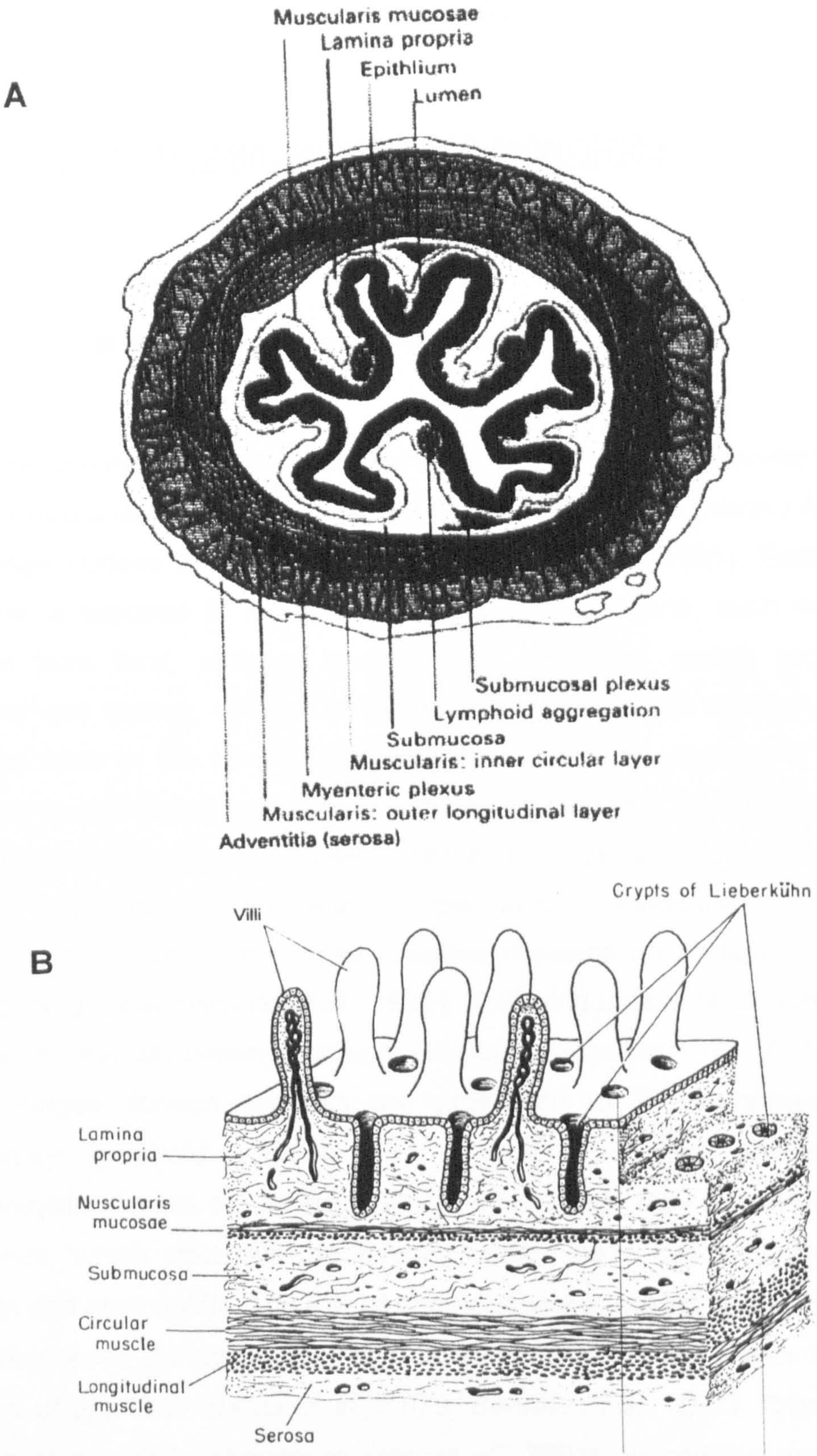


Figure 1.4 – These diagrams show the different mucosal layers of the gastrointestinal wall. **A**- transverse section of the small intestine (from Burkitt, *et al.*, 1993) and **B**- is a stereo diagram of the mucosal surface & submucosa (from Brook *et al.*, 1980).

mechanisms. Goblet cells are the cells that specialise in producing mucus, which is an important part of this defence (Ishikawa *et al.*, 1994).

1.7 – INTESTINAL IMMUNITY TO HOOKWORMS

1.7.1 – General Aspects of Mucosal immunity

The intestine with its mucosal structure, plays an important roles in immune responses against any invaders in addition to its primary function as absorption surface of the nutrients (Field and Frizzel, 1991). Each day, the intestine is exposed to many different types of antigens, such as antigens derived from food, resident bacteria and epidermal growth factor (EGF) (Weaver and Walker, 1988). The lumen of the intestine, in common with other mucosal sites in the body cavity such the respiratory system, is subject to invasion by pathogenic organisms such as hookworms. In order to mount an immune response against these potential pathogens, the mucosal immune system must survey antigens in the lumen. Evidence suggests that immunosurveillance by the small intestine depends on transport of antigens across the gut wall (Winner *et al.*, 1991). Following uptake of foreign molecules and cells, and processing through antigen presenting cells (APC) such as macrophages, foreign epitopes are presented to T lymphocytes in local secondary lymphoid organs. After clonal expansion, antigen specific lymphocytes migrate out to the mucosal sites where they release appropriate cytokines, which influence the effector cells. These cells are mast, goblet, Paneth and eosinophils. The role of each cell type is not yet fully understood but their responses during parasite or helminth infections have been documented (Ackert *et al.*, 1939; Befus *et al.*, 1979; Behnke *et al.*, 2003; Creamer, 1967; Gause *et al.*, 2003; Theodoropoulos *et al.*, 2001). In addition to these cells, there are other factors that help to control infections on first exposure, such as natural killer (NK) cells, complement system and interferons. The mucosa also contains proteins that constitute the innate humoral factors, which are secreted

onto the mucosal surfaces by exocrine glands. They are able to carry out their protective functions independent of the presence of specific antibodies. These are the proline-rich proteins (PRP) and histadine-rich proteins (HRP) (Hey and Moreno, 1989; Oppenheim, 1989).

Other features of the gut environment that may regulate parasitic infections are alterations in intestinal structure during infection. These involve changes in the villous height and crypt depth with shortening of the villi and crypt hyperplasia. Such changes may reduce the ability of worms to attach to the intestine

1.7.2 – Mucosal immunity to hookworms

Parasitic nematodes, such as hookworms, invade the host as L3s. These stages then moult to L4 and on arrival in the intestine reside there for many months as adults (Schad, 1991).

The mucosal immune system in the intestine is the most important barrier to infection and thus, acquired resistance to infection in the gastrointestinal tract is mediated through the mucosal immune system (Befus, 1990). Parasitic nematode elicit responses in the host that results in their spontaneous expulsion from the intestine during the enteric phase of primary infection (Lawrence, 2003). This has been referred to as a "spontaneous cure" and is known to be T cell mediated (Wakelin and Denham, 1983). Spontaneous cure is very efficient in rats and mice and takes 10-15 days to expel worm from intestine (Kamal, 2001). The effector mechanism against GI nematode appear to involve antigen-specific T cell response which induce antibody response and inflammatory changes, with the release of a plethora of chemical mediators ultimately leading to the expulsion of the worms (Befus, 1995). This include the Th2 cells which mediate protective immunity to intestinal nematodes through IL-4, IL-5, IL-9 and IL-13 (Shea_Donohue *et al.*, 2001) and (reviewed in Gause, *et al* 2003). A delayed expulsion occurs in immunosuppressed hosts, and complete absence of expulsion occurs in T cell-deficient hosts (Ruitenberg *et al.*, 1977) or T cell-deprived hosts (Kamal, 2001). On the other hand,

accelerated expulsion was found in hosts immunized, given immune serum or given T cells from immune hosts (Grencis *et al.*, 1985; Robinson *et al.*, 1995b).

Studies from murine model systems infected with GI nematode, such as *T. spiralis*, showed that there is an acute inflammatory response in the small intestine. There is a close correlation between the loss of the worms and the onset of the inflammation, as suppression of the inflammatory responses prevent worm expulsion (Na *et al.*, 1997; Parmentier *et al.*, 1989). The inflammation is characterised by changes in the intestinal architecture, such as villus atrophy and crypt hyperplasia, and changes in the infiltration of the immune cells including mastocytosis, goblet cell hyperplasia, eosinophilia and high levels of antibodies (Miller and Jarrett, 1971; Robinson *et al.*, 1995a).

Resistance of mice to infection by hookworms probably involves a combination of innate and immunologically mediated mechanisms. The murine environment may be recognised as unsuitable for development, thus triggering arrested development in the *Ancylostoma* spp. In fact, all hookworm species fail to grow and establish normally in the intestines of mice but the precise factors restricting their success in this host are still unknown.

However, limited development of hookworms has been demonstrated in hamsters. *A. ceylanicum* not only establishes in hamsters but grows and reproduces in this host (Norozian-Amiri and Behnke, 1993). Such infections can last for many months, supporting the validity of using the hamster model as a model of chronic hookworm infection. Another species, which can develop in hamsters, is *N. americanus,* but this laboratory model is complicated by the failure of this parasite to successfully infect adult animals. Instead infections are given to neonatal hamsters and only when infected in the first 4 days of life, will hamsters eventually support an adult worm infection. Two possible explanations could account for this observation. The first is that the larvae fail to complete the migration through the lungs to the gut in adult hamsters because of innate barriers and the other is that specific immune responses may be involved (Behnke and Pritchard, 1987).

The mucosal immune system is based on mucosal associations of lymphoids tissue (MALT). These include lymphoid tissues found in the gut

(GALT) and bronchus associated lymphoid tissue (BALT). The IgM and IgG antibodies titre was observed in dogs infected with *A. ceylanicum* by Carroll and Grove (1984). They found that there were no significant correlations between the intensity of the infection and IgM and IgG titre antibodies two weeks after infection. However, a significant positive correlation between exposure to increasing numbers of infective larvae and increasing titre of IgG antibody was observed six weeks after infection. Also, a specific anti-hookworm Immunologlobulin A (IgA) was measured in dogs with *A. ceylanicum* infection by Carroll and Grove (1985). They reported an increase in the level of IgA antibodies in the intestine three weeks after infection. Work by Garside and Behnke (1989) found that the levels of anti-parasite antibody rise after a week post infection until reaching a peak at seven weeks.

Additional to the previous factors, there is a large population of leucocytes in the epithelial sites such as the intestine and respiratory tract. These intraepithelial leucocytes (IEL) comprise some neutrophils, eosinophils, macrophages, mast cells or basophils under normal conditions, although in helminth infection and some other associated inflammatory responses, mast cell hyperplasia and a local eosinophilia can occur in the epithelium. Since little is known about the existence or even role of IELs during hookworm infection, this is an important area for future work.

1.8 – INTESTINAL HISTOPATHOLOGY

The small intestine is very important to the host as the fundamental site for taking up food products (Brooks and Paynton-Brooks, 1980). The intestinal architecture has a distinct shape that provide a big surface area in order to be able to absorb as much as possible of the food elements (Figure 1.5)(Campbell *et al.*, 1999). Inside are finger-like villi and underneath then crypts of Lieberkuhn. The integrity of this layer in health is maintained despite the fact that the whole surface is shed and replaced every 2-3 days. This layer undergo regeneration and is able to repair any damage caused by harmful materials including parasites (MacDonald, 2003). Hookworms have been suspected as

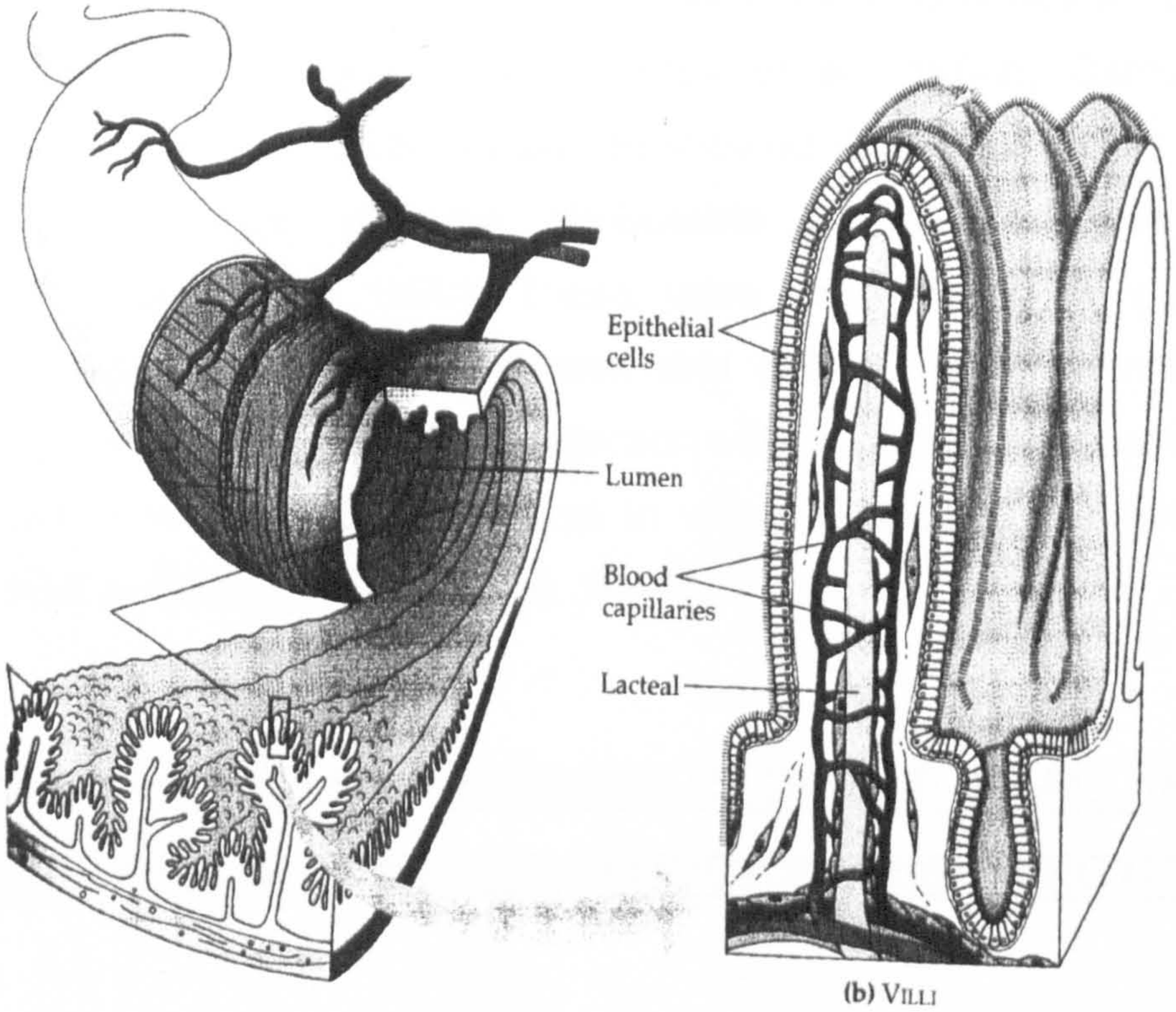


Figure 1.5 – Intestinal architecture showing villi and crypts of Lieberkuhn of the intestine (Taken from Biology, *Campbell, et al.*, 1999).

causing severe intestinal damage and therefore the ability of the host to survive is dependent on rapid repair (Behnke, 1991).

The mucosal layer of dogs infected with *A. caninum* showed little evidence of any damage with minor inflammatory changes recorded at the sites of attachment (Verma *et al.*, 1968). The heights of villi and the depth of the crypts of the neighbouring tissue were normal and there was no evidence of significant vascular or tissue hemorrhage. However, these changes were marked in dogs infected with *A. ceylanicum* (Carroll *et al.*, 1984b; Carroll *et al.*, 1985). Investigations of human intestines showed little evidence of more than minor histopathological changes attributable to the presence of hookworms (Pimparker *et al.*, 1970). These were seen during *A. duodenale* and *N. americanus* in humans (Banwell and Schad, 1978; Burman *et al.*, 1970). However, in cases of severe infection with *A. duodenale*, marked changes were seen at the level of shortened in villi and cellular infiltration at the site of infection (Salem and Truelove, 1964)

1.9 – CELLULAR COMPONENTS OF THE SMALL INTESTINAL EPITHELIUM INVOLVED IN HOOKWORM INFECTION

The epithelium of the small intestine originates from crypt stem cells, which give rise to four different cell lineages (Cheng and Leblond, 1974). These are absorptive enterocytes, enteroendocrine cells, goblet cells and Paneth cells. The last three types of cells proliferate, differentiate then migrate from the crypt and upwards towards the villous tip where the cells are sloughed off into the lumen in a well-organised continuum. These cells are known to be regulated and respond differently to different kinds of infections in the animals' intestine. Other studies by Banerjee, *et al.* (1970) showed mild cellular infiltration into the intestines of mice infected with *A. caninum*. Furthermore, investigation of some accessory cells by Carroll *et al.*, (1986) in hookworm infection included mast cells and eosinophils, which are believed to play an important role in regulating parasitic infection, particularly nematode infections.

The differentiation and proliferation of the intestinal epithelial cells has been shown to be regulated by many factors, such as, transforming growth factor-α and β (Mahida *et al.*, 1992) and components of the extracellular matrix (Carroll *et al.*, 1988). Furthermore, it was found that INF-γ, which is released by antigen-activated TH1 cells, plays a role in epithelial proliferation. The depletion of INF-γ in SCID mice during infection with *Trichuris muris* resulted in no significant increase in epithelial cell proliferation and effectively prevented the development of crypt hyperplasia without altering infection outcome (Artis *et al.*, 1999). However, it is still unclear which factor/s regulate the commitment of crypt cells to differentiate along particular cell lineage.

1.9.1- Mast cells

Paul Ehrlich (1878) first noticed mast cells when he saw that certain cells stained metachromatically (red) with a blue-violet dye. Mast cell numbers changed in abundance in relation to infection and some pathological conditions. Unna (1984) discovered the fact that lesions of urticaria pigmentosa consist almost exclusively of mast cells and were associated with pathologic conditions. Mast cells were recognised as a source of tissue-derived inflammatory mediators by Ryan and Majno (1977).

It was Maximov (1906) who first described the gut mucosal mast cells in the jejunal villi and submucosa of the rat. While histological changes in the intestine of *Nippostrongylus* infected rat were noted by Taliaferro and Sarles (1939) they also stated that the connective tissue basophils and globule leukocytes increased when the parasites were expelled during infection. There was a long-running dispute about the existence of mast cells in tissue, where some authors saw them and other did not, until Enerback (1966b, 1966c) showed that the mucosal mast cells represent a distinct cell lineage. This was found after a systematic study of the fixation and staining of rat mast cells in different parts of the gastrointestinal tract. Mast cells have since been subdivided into two subpopulations, i.e. Mucosal Mast Cells (MMC) and Connective Tissue Mast Cells (CTMS). MMC are present in the mucosa of the

respiratory system and the digestive tracts, whereas CRMS are dispersed in the connective tissue (Dehlawi, 1986; Hopkins and Smith, 1997).

Scientists have since described the differences between different types of mast cells. It is becoming increasingly clear that mast cells in the mucosal layers are very different from those elsewhere in terms of their tissue distribution, staining characteristics and the proteases they contain. Table 1.1 summaries some of the differences between the two sub-populations of mast cell, which suggest different functional roles *in vivo*. They also differ between species, individuals and sites in an individual (Lee *et al.*, 1985).

Infiltration of the intestinal mucosa by mast cells has been found to be associated with many helminthic infections and represents a key component of the host-parasite relationship in the gastrointestinal tract (Befus *et al.*, 1986; McDermott *et al.*, 2003; Wells, 1962). It was established that mast cells respond by an increase in the total number during infection with nematode parasites such as *N. brasiliensis* and *T. spiralis* (Dehlawi, 1986; Knight *et al.*, 2000; Nawa and Korenaga, 1983; Wells, 1962). Work by Ruitenberg and Elgersma (1976) showed that mice and rats infected with *T. spiralis* or *N. brasiliensis* demonstrate an increase in the number of mast cells in the mucosa of the small intestine. Other workers showed that the mast cell response (known as mastocytosis) can be delayed or is less marked in the case of mast cell-deficient mice, W/W^v mice infected with *T. spiralis* (Alizadah and Murrell, 1984). Worm rejection takes longer in cases of the same W/W^v mice infected with *N. brasiliensis* (Crowle and Reed, 1981) but infections were only marginally extended, suggesting that in mice, mast cells do not play a prominent role in resistance to *N. brasiliensis* (Ha *et al.*, 1986; Mitchell and Wescott, 1983; Uber *et al.*, 1980). In contrast, infections with *S. ratti* and *S. venezuelensis* are generally extended (Khan *et al.*, 1993). Japanese workers concluded that mastocytosis was vital for resistance to *Strongyloides* species. It is also generally concluded that the expulsion of *T. spiralis* is dependant on mast cells (Ha *et al.*, 1983; Knight *et al.*, 2000; Miller, 1971b; Miller, 1971c; Miller and Jarrett, 1971a; Urban *et al.*, 2000). This was recently confirmed by studies of McDermott, *et al* (2003) who found that mast cells are directly responsible for increasing epithelial permeability in the intestine of mice infected with *T. spiralis*

Table 1.1 - Subpopulations of mast cells, mucosal mast cells (MMCs) and the connective tissue mast cells (CTMCs). The differences in their morphology and pharmacology suggest different function roles *in vivo*.

	MMC	CTMC
Location *in vivo*	Gut and lung	ubiquitous
Life span	<40 days (?)	>40 days (?)
T-cell-dependant	+	-
Number of Fc€ receptors	25×10^5	3×10^4
Histamine content	+	++
Cytoplasmic IgE	+	-
Major AA metabolite LTC_4: PGD_2 ratio	25:1	1:40
DSCG/theophlline inhibits histamine release	-	+
Major proteoglycan	Chondroitin sulphate	Heparin

Taken from: Hopkins and Smith, (1997).

and that mast deficient in mast cells-specific protease fail to increase intestinal permeability and therefore fails to expel the worms. These studies have led to the current hypothesis that the effectors of worm expulsion vary between hosts and parasites. In rats infected with *T. spiralis,* mastocytosis is temporally associated with the immune expulsion of the adult worms (Alizadeh and Wakelin, 1982), whereas in the case of *N. brasiliensis,* mastocytosis is frequently observed to occur after worm expulsion has been completed (Keller, 1971; Kelly and Ogilvie, 1982; Newlands *et al.*, 1990; Wells, 1962).

Mast cells were also observed in humans during hookworm infections but their relevance is still not clear and much further work is required to clarify the role of MMC in humans in relation to GI nematodes (Loukas and Prociv, 2001). Carroll *et al.* (1986) and studied comprehensively the kinetics of various cells in the intestine of mice infected with *A. ceylanicum* and showed pronounced increases in *lamina propria* mast cells and goblet cells but hookworms do not mature in mice and therefore this model is not totally relevant to infections in humans. Overall, relatively little is known about mast cell responses during hookworm infections, particularly in humans. However, studies in hamsters infected with helminth infection (Behnke *et al.*, 1994; Shi *et al.*, 1994) and hookworm (Garside, 1989) revealed an increased number of mast cells in the mucosa of the small intestine and there is evidence for a role in protection against some parasites, such as *T. spiralis* (Gause *et al.*, 2003; Knight *et al.*, 2000).

1.9.2 – Goblet cells

Goblet cells are highly polarized exocrine cells recognised by their apical accumulation of secretory granules. They arise by mitosis from multipotential stem cells at the base of the crypt (Cheng and Leblond, 1974b) or from poorly differentiated cells in the lower crypt. Goblet cells migrate from stem cell zones when they are immature, their morphology changes, then they diminish in size from a pyramidal immature cell to a characteristic goblet cup-like shape (Specian and Oliver, 1991). Goblet cells secrete a heterogeneous collection of

high-molecular-weight glycoproteins that can be separated into multiple mucin species by ion-exchange chromatography (Forstner, 1978; Specian and Oliver, 1991). It has been noted that carbohydrates in humans and rats comprise 80-85% of the mucin is molecular weight (Fahim *et al.*, 1983; Wesley *et al.*, 1985). The majority of the protein core is composed of serine, threonine and proline amino acids. LaMont and Isselbacher (1975) found that in pathological cases, such as the human colon cancer, goblet cell mucins may change in carbohydrate content. Whereas patients with ulcerative colitis, the qualitative alterations in the mucins were both secreted and retained (Podolsky *et al.*, 1993), which may cause the epithelium to become more vulnerable to invasion by certain pathogens, or could increase the protection of the epithelium and though protecting the host from nematode infection (Miller and Nawa, 1979b; Onah and Nawa, 2000). This secretion forms a mucin blanket, which contains water, electrolytes and antibodies, over the epithelial layer. It provides physical, chemical and immunological barriers to protect the epithelium from luminal pathogens and lubricates the intestinal epithelium.

Mucin secretion by goblet cells is believed to be a continuous process that maintains a mucus blanket overlaying the mucosa (reviewed in Specian and Oliver, 1991). Moreover, it is assumed that the cytoskeleton of a goblet cell plays a part in regulating this baseline secretion.

Microtubules have been implicated in directing granule migration to the cell apex, while the actin filaments are thought to prevent contact of the granule membranes with the cell's membranes and therefore, limiting mucus secretion (Specian and Oliver, 1991). Goblet cells have been found to secrete most of their contents in response to stimulation with cholinergic agonists, chemical and physical irritation or intestinal anaphylaxis. Other secretory products, such as IL-7 and intestinal trefoil factor have also been found in goblet cells (Podolsky *et al.*, 1993). Studies investigated goblet cell changes in the intestine during some helminth infections indicated that goblet cells release their contents when they became activated by the trigger of T cells (Ishikawa *et al.*, 1994) and particularly with a switch of Th2 cytokines (Ishikawa *et al.*, 1997).

1.9.3 – Paneth cells

Schwable (1872) was the first to discover Paneth cells and Paneth provided a further description in 1888. They are found in the base of the crypts of Lieberkuhn in a variety of different species including humans (Satoh *et al.*, 1990), but not in carnivores and artiodactyls (Meyer *et al.*, 1970; Paneth, 1888). Although Paneth cells are found in the normal small intestine and appendix, they have been reported to be most abundant in the region of the ileum (Herzog, 1937). They have also been found in many disease states such as: appendicitis, ulcerative colitis, Crohn's disease, carcinomas of the caecum and rectum, and the large intestinal adenomas and papillomas (Lewin, 1969a).

Paneth cells are characterised by their eosinophilic, zinc-binding protein-containing granules (Sawada *et al.*, 1994) which reside in the apical cytoplasm. They have additional features which include their extensive Golgi complex and endoplasmic reticulum (Mathan *et al.*, 1987). The ultrastructure of Paneth cells has been found to be different from one species of host to another. Satoh *et al.*, (1990) reported two morphologically distinct Paneth cell granules. In monkeys, echidna, moles, bats, and man, there is a highly electron-dense matrix which is almost homogenous, whereas in the mouse, horse, rat and golden hamsters, the granules are bizonal with an electron-dense core and an electron-lucent peripheral halo (Satoh *et al.*, 1990; Takehana *et al.*, 1998). Lysozyme has been detected in the electron dense granules and minimally in the surrounding pale halo (Mathan *et al.*, 1987). It was further found that the number, size and electron density of these granules increased in those cells which are closer to the base of the crypt (Mathan *et al.*, 1987).

Mathan *et al.* (1987) noted that human Paneth cells contain large mucus-like granules in addition to eosinophilic and highly electron-dense granules. It has been found that these mucus-like granules are characteristic in their carbohydrate contents (Leis *et al.*, 1997). The positive reaction between the electron dense cores in the equine Paneth cells and the lectin indicated that they contain carbohydrates (Takehana *et al.*, 1998). In equine Paneth cells a

different carbohydrate composition has been found in the peripheral halo of the granule compared to the dense core (Takehana *et al.*, 1998).

Paneth cells are found to contained many different factors such as tumour necrosis factor (TNF- α) (Tan *et al.*, 1993), lysozyme (Peeters and Vantrappen, 1975), and epidermal growth factor (Poulsen *et al.*, 1986). Accordingly, Paneth cells have various activities attributed to their range of secreted components. Some of these functions are the secretion of digestive enzymes (Bohe *et al.*, 1984; Senegas-Balas *et al.*, 1984) or substances which affect proliferation and differentiation of the intestinal epithelium (Creamer, 1967; Poulsen *et al.*, 1986). It has been found also that these cells have a role in the elimination of heavy metals (Elmes, 1976; Phillpotts, 1984) and controlling the bacterial milieu in the intestine by releasing antibacterial substances called cryptdins (Ouellette *et al.*, 1989; Rodning *et al.*, 1982; Rodning *et al.*, 1976).

Paneth cells vary in their response to infections from one animal to another and from one infection to another. There is data from several studies that shows that Paneth cells degranulate in response to live and heat-inactivated bacteria present in the intestinal lumen (Satoh *et al.*, 1986). As these cells release their antimicrobial contents in response to bacterial infection, it is thought that they have a protective function in the intestine. Data, which will be presented later in this study will show a reduction in numbers of Paneth cells during infection. This is similar to that observed in adoptively immunised mice during challenge infection with *Eimeria vermiformis* (Rose *et al.*, 1992). The reduction of Paneth cell numbers could be attributed to degranulation resulting in the subsequent release of material into the crypt lumen and hence poor staining qualities using the reagents.

1.9.4 – Eosinophils

Tissue eosinophils are characteristic of hookworm and many other parasitic infections in man and animals. It has long been considered that eosinophils play an important role in defence against metazoan parasitic organisms (Kazura, 1980). This suggestion was supported by the development

of the anti-eosinophil antisera (Mahmoud *et al.*, 1973) which were used to study the host-parasite relationship in eosinophil-depleted mice infected with *T. spiralis* (Grove *et al.*, 1977). This study concluded that eosinophils contribute to resistance to the systemic phase of trichinosis.

Increases in mast cells and eosinophils have long been recognised to be a characteristic feature of infection with helminth parasites. Initially, it was thought that eosinophils act as regulatory cells to inhibit the inflammatory reactions caused by mast cell degranulation. However, this hypothesis was reviewed after research carried out by Butterworth (1984) who found that eosinophils could kill the early stages of *Schistosoma mansoni* in *in vitro* cultures. Subsequently, other studies involving other species of nematode parasites of human and veterinary importance, demonstrated that eosinophils play a key role in killing parasites *in vitro* (Butterworth, 1984; Butterworth and Thorne, 1993; Rainbird and *al*, 1993). The eosinophil-mediated killing was, in the majority of cases, most effective against the larval stages. This required co-operation with antibody and/or complement for maximal killing capacity. Furthermore, helminth activated eosinophils were most efficient at killing parasites *in vitro* (Butterworth and Thorne, 1993; Rainbird *et al.*, 1998).

The role of eosinophils in parasite resistance *in vivo* was supported by the histological observations of damaged or dying parasites in host tissues surrounded by eosinophils reviewed earlier (Butterworth, 1984; Butterworth and Thorne, 1993). Although the association between eosinophils and dead or damaged parasites was shown in many studies, it was not always clear if these associations were present at the time of parasite killing or were a consequence of parasite death and/or tissue damage. However, studies examining cells and products during the immune response showed that there was an intense infiltration of eosinophils in the skin lesion of patients infected with *Onchocerca volvulus* (Ackerman and *al*, 1990). Seitz *et al.*, (1987) also showed concentration of eosinophils around damaged schistosomula in the skin of immunized baboons. Furthermore, immune rats showed clusters of eosinophils around larvae of *Strongyloides ratti* (Moqbel, 1980)

The importance of eosinophils as potential effector cells for the elimination of helminth parasites has been demonstrated in *in vitro* cultures (Meeusen and Balic, 2000). However, their role in *in vivo* has been more difficult to establish. It has been showed in early work that there are some associations between damage or dead parasites in histological sections and eosinophils (Butterworth, 1984). Moreover, resistance to the nematode *Trichostrongylus colubriformis* Romney Lamb and the number of blood eosinophils induced after infection correlated significantly (Buddle *et al.*, 1992). However, more recent studies, using mice that have reduced or increased eosinophil levels, through the targeting of the IL-5, have not supported an *in vivo* role for eosinophils in resistance to parasites (Harndon and Kayes, 1992).

1.10 – IMMUNOSUPPRESION AND CYCLOSPORIN A (CsA)

One way to study the component of the immune system is to inactivate the ability of the host to mount a fully competent response. This can be achieved in various ways, including surgery, ablation of key organ such as the thymus and by treatment with drugs that interfere with the immune response either generally in a non-specific manner or more specifically by affecting a particular population of cells thought to be involved in immunity. A more modern approach is to use knockout animals, which have key gene inactivated or transgenic animals that may show enhanced immunological activity of particular components (e.g. IL-5).

Some candidate effectors, such as mast cells, are known to be initiated by T cells. Therefore, scientists found immunosuppressive drugs or agents in order to block these and their role in immune responses. Many drugs were found to be useful in this field including cortisone (Norozian-Amiri, 1993), FK506 (Toyota *et al.*, 1996) and Cyclosporin A (Batiuk *et al.*, 1996). The later was used in many model systems and in treatment of humans and found to be very effective in organ transplantation in facilitating the survival of grafted tissue (Mowat and Ferguson, 1982; Vonfliedner *et al.*, 1982).

CsA is a natural hydrophobic cyclic polypeptide consisting of 11 amino acids, thought to inhibit the activation of T cells by interacting with the intracellular route of signalling following ligation of the T cell receptors (TCR) (Damoiseaux *et al.*, 1997) or by inhibiting transport to the nucleus of the cytoplasmic component of the transcription factor (NA-AT) (Oran *et al.*, 1997). More specific immunosuppressive action of CsA depends on the drug binding to the intracellular receptor cyclophilin (CyP) (McLauchlan *et al.*, 2000). The resulting CsA-CyP complex inhibits the Ca2+ regulated protein phosphatase calcineurin and down-regulates signal transduction events (McLauchlan *et al.*, 2000). However, the exact molecular events are still controversial (Cummins *et al.*, 1989) but CsA is known to prevent the production of cytokines, such as Interleukin-2 (IL-2) (Thomson and Webster, 1988). It is also thought that this chemical blocks degranulation as well as the production of cytokines. The role of this drug during parasitic infections has been investigated in many different models (Bolas_Fernandez *et al.*, 1988; Roberts *et al.*, 1995; Roberts *et al.*, 1997). However, nothing is known about its role during hookworm infection.

1.11 – THE AIM OF THIS STUDY

The available literature about the relationships between the hosts and their parasites indicated that this is still an area of much interest and controversy. There are still major gaps in our understanding and much scope for further investigation. The host-parasite relationships of hookworms in particular are poorly understood, mainly because of a lack of suitable animal models.

Hookworms are not dissimilar to some of the popular models investigated and their life cycles and histology have close parallels in *Nippostrongylus brasiliensis* and *Heligmosomoides polygyrus*. However, they also have distinct characteristics that distinguish them from these models.

The overall objective of this project was to provide basic and vital information facilitating a more thorough understanding of the immunological, inflammatory and pathological processes, which occur during hookworm

infection and particularly in *A. ceylanicum* in the hamster laboratory model system. Also to distinguish between those processes which are involved in protecting the host from infection and those, which enable parasites to survive. The present study was designed to investigate the mucosal and cellular immune response within the hamster intestine infected with *A. ceylanicum.*

The objectives of this research include:

1)- Quantification of changes in the intestinal architecture during infection with hookworm under different infection regimes.

2)- Quantification of the associated cellular changes with a particular emphasis on cell types known to be associated with resistance to infection, e.g. mast cells, goblet cells, Paneth cells and eosinophils.

3)- By exploiting a range of experimental protocols, such as primary infection, challenge, trickle infection and immunosuppresion, in some of which the host shows resistance to infection, and in others where no resistance is evident, to elucidate those changes which are related to host protection and those which facilitate parasite survival.

4)- Finally, it was hoped that by conducting this study a contribution could be made enriching the literature on host-parasite relationships in respect of the host defence components involved during hookworm infection in man and other domestic animals. It is also hoped that this information will be useful to the further understanding of human hookworm disease and will lead to further work on the host immune defence against hookworms.

CHAPTER TWO

GENERAL MATERIALS AND METHODS

2.1 – ANIMALS

2.1.1 – Hamsters

Golden hamsters (DSN strain) were originally obtained from Harlan Olac in 1983 and since then have been maintained in the animal house of the SLES as a closed colony. Animals were kept under conventional animal house conditions. Pelleted food and tap water were supplied *ad lubitum*. All cages were cleaned twice weekly and all experimental animals were regularly monitored for weight changes. Animals were first weighed one or two weeks before infection and thereafter twice weekly until the completion of each experiment.

Since the colony was maintained under conventional animal house conditions, and not specific pathogen free, the animals were exposed to various micro-organisms present in the animal house environment. In order to prepare hamsters for infection and reduce other competing intestinal micro-organisms, all animals allocated to experiments were pretreated for one week with Emtryl (see appendix 2.6.1), then for another week with Terramycin (see appendix 2.6.1), both given in the drinking water, and were rested for one week on normal drinking water prior to infection.

2.1.2 – Mice

Mice of the NIH strain were purchased from Harlan-Olac Ltd., Bicester, Oxon, UK at eight weeks of age and were kept in groups of five under conventional animal house conditions. Food and water were supplied at all times *ad lubitum* and the bedding was changed twice weekly.

2.2 – PARASITES.

Two species of parasitic nematodes were used throughout this work. Most of the work is based on the canine hookworm *Ancylostoma ceylanicum* but some experiments also incorporated groups infected with *Trichinella spiralis*.

2.2.1 – Maintenance of *Ancylostoma ceylanicum* and larval recovery.

Previously infected hamsters (usually between 14-30 days earlier) were placed in a grid-bottomed cage and suspended over trays containing moist tissue paper for approximately 24 hours. Faecal samples were collected and brought into the laboratory and then mixed with an approximately equal amount of granulated charcoal. Fungizon (Amphotericin B, 250 g/ml, GibcoBRL), peat and distilled water were added to form a paste. The mixture was then heaped in the centre on damp 11 cm filter paper, which were then placed in a 9 cm plastic Petri dish. Each Petri dish was placed in a square plastic box then sealed by a lid, held down securely with masking tape and incubated for 6-10 days at 26-29°C. This period was sufficient to enable the larvae to complete their development to the infective L3 stage. Infective larvae generally ascended from the feacal slurry onto the lid of the Petri dish where they accumulated. These larval were then washed off the lid with distilled water and collected into a beaker.

2.2.2 – Maintenance of *Trichinella spiralis* and larval recovery.

The strain of *T. spiralis* used throughout this project was originally obtained from the London School of Hygiene and Tropical Medicine and has been maintained by regular passage in outbred CFLP mice since 1976. Infective stage larvae were obtained by digestion of muscle tissue from stock mice, which had been infected for at least sixty days. The procedure involved

killing one stock mouse then skinning, mincing and digesting the tissue for 2-3 hours in a digestion solution (see appendix 2.8.2 (U). The digest was poured into a measuring cylinder and left for approximately 10-15 minutes to enable larvae to settle on the bottom. After setting, larvae were separated from the remaining tissue by passing the digest mixture through a gauze and were collected by washing the gauze through with prewarmed PBS. The larvae were eventually suspended in prewarmed 0.2% agar in order to keep them in a suspension, to enable reproducible counts on aliquots taken for estimation of their concentration and eventually for infection. Experimental and animals for passage were infected orally using a syringe with a blunt-ended needle (Gavages needle).

2.3 – PARASITOLOGICAL TECHNIQUES.

2.3.1 – Recovery of *A. ceylanicum*.

The small intestine was removed from infected animals after euthanasia in chloroform, opened longitudinally and was placed into a large Petri dish containing Hanks' saline. Petri dishes were kept on hotplates at 37° C for 4-6 hours to enable all worms to detach from the mucosal tissues. Worms were then picked out with the help of fine forceps and were placed in Petri dishes in Hanks' saline to enable them to be counted.

2.3.2 – Recovery of *Trichinella spiralis*

Animals were killed with an overdose of chloroform and the entire small intestine was removed and split longitudinally. It was placed in the centre of a nylon gauze and submerged in 40ml of prewarmed (37-40° C) Hanks' saline in a 50ml beaker. Intestinal samples were removed after incubation for 2-3 hours

and the worms were poured into a large Petri dish and then counted, after sedimentation, under a dissecting microscope.

2.3.3 – Preparation of *Ancylostoma ceylanicum* larvae for infection.

Fresh third stage larvae of *A. ceylanicum* were placed into a universal tube and suspended in distilled water. The required number of larvae was estimated in 0.2ml aliquots taken from the larval suspension. Each 0.2ml sample was evacuated from the syringe, drop by drop, onto a clean Petri dish, and the larvae in each drop were counted under a dissecting microscope to give the total. If the number of suspended larvae in a 0.2ml sample did not reach the required concentration for infection, the larvae were sedimented for longer, an appropriate volume of supernatant was removed to concentrate the remaining larvae and more aliquots were counted to confirm that the required concentration had been achieved. Alternatively, a suitable volume of water was added to dilute the larval suspension. A. ceylanicum is a pathogenic nematode that induces dose dependant anaemic in hamsters and infections can be lethal if the hamsters can not cope with the number of worms established in the intestine (Behnke, 1990). It is therefore necessary to be extremely careful in choice of the dose of larvae. The doses of larvae which were chosen for infection of animals were based on knowledge from previous works that indicated a good responses by the mucosal immune system within the period of study. Hamsters tolerate these levels of infection that were used with a minimal stress in accordance with the Animal Procedure Acts (1986). When observation of the parameters in the hamsters were intended to be left for more than 20 days after initial infection, doses did not exceed 100 L3 but rather remained in the range between 50-100 L3.

2.3.4 – Preparation of larvae of *T. spiralis* for infection

T. spiralis larvae were harvested and collected into a small beaker. Larvae were suspended in 0.2% agar and the total volume was adjusted to give the required dose in 0.2 ml. Animals were then infected orally using a syringe with blunt gripped gavages needle.

2.3.5 – Estimating hookworms-egg concentration in faecal samples

Approximately 1 g of faeces from hamsters infected with *A. ceylanicum* was collected into a universal tube. The amount taken was weighed carefully to the nearest 1g of faeces. 10ml of saturated sodium chloride solution were added to each sample and mixed thoroughly and left for approximately 2-3 hours on a magnetic stirrer. The mixture was agitated and poured quickly through a sieve and washed through with a further 50mls of the same solution (saturated sodium chloride). Hookworm eggs were then counted after floatation in a standard McMaster helminth egg counting chamber. Six counts were made on each sample and averaged to give the mean value. The concentration of eggs in faeces was expressed as the number of eggs per gram of faeces (EPG).

2.3.6 – Measurements of Worm length.

Worms for measuring were kept in Hank's saline if they were to be measured on the day of collection. However, if measuring was delayed they were preserved by the addition of 1ml of a mixture of formalin/ethanol (v/v 50:50) and worms were kept in this solution until they could be measured. They were drawn with the aid of a Camera Lucida and these drawing were measured using a bit-pad digitiser linked to a PC. All drawing was traced twice and the

mean length was calculated. The sex of worms was also recorded if the worms had reached late L4 or adult stages at which they could be sexed reliably.

2.4 – HISTOLOGICAL TECHNIQUES.

2.4.1 – Fixation of tissues and preparation of tissues for staining

At autopsy, four pieces of tissue were taken from the intestine (each about 1 cm square) approximately 10 cm below the pyloric sphincter. Each one centimetre section of the intestine was destined for different treatment. The four fixatives used were "Carnoy's, 10% formalin, Clark and MT" (see appendix for the details of each fixative). Carnoy's-fixed samples were kept from 4-6 hours in the fixative and then transferred to 70% ethanol solution. Samples fixed in Clark and MT fixatives were kept for approximately 24 hours in the fixatives and then moved to 70% ethanol. Formalin-fixed samples were kept in formalin until processing.

Tissues fixed in Carnoy's, 10% formalin and MT Fixatives were washed thoroughly with 70% ethanol solution then placed in plastic wire cassettes and dehydrated using pre-programmed Electronic SHANDON Citadel Histokinette. The histological processor passed the tissue through 6 changes of ethanol (70%, 80%, 90%, 95%, and 2 x absolute ethanol) followed by three changes of xylene and finally two changes of paraffin polymer (polywax, Tissue-Tek II, Sakura, Code: 4653) at 56°C. Samples remained in each solution for one hour before passing on to the next treatment. Tissues were embedded in pure paraffin wax, left to dry, then mounted on small wooden blocks and sectioned at 6µm using a microtome (Reichert-Jung, Microtome 2050 Supercut, Cambridge Instruments GmbH) Samples fixed in Clark's fixative (See appendix 2.8.2 C) were attached onto small pieces of paper to maintain their evenness, any large intestinal digesta were gently removed, the samples were washed and stored in 75% ethanol until required for sectioning.

2.4.2 – Staining tissue sections.

Sections were deparaffinised by heat to 50°C on a wired hotplate, followed by 5 minutes in each of two xylene washes followed by two minute washes in each of absolute ethanol, 90%, 80%, 70% and 50% then finally dipped into distilled water. Sections were then stained according to the requirements of the cell type to be quantified (see appendix for recipes of stains used)

2.4.2.1 – Staining for mast cells (fixed in Carnoy's).

Sections of samples fixed in Carnoys were placed into a coplin jar full of prewarmed (50°C) Alcian blue and left for 45 minutes. The tissue was then counterstained by immersion in another jar full of prewarmed Safranin O for 5-10 minutes. The slides were then dehydrated by a rapid dip through different grades of ethanol starting from distilled water and through to absolute ethanol for not more than 1.5 minutes in each. Sections were transferred through fresh xylene 3 times for clearing, and then mounted using DPX mounting solution. (See appendix for recipe of stain used).

2.4.2.2 – Staining for goblet cells (fixed in 10% formalin).

Sections were dipped into Alcian blue (pH 2.5) for 5-10 minutes then washed in distilled water to remove excess stain and treated with 1% periodic acid for 5-10 minutes. Sections were then washed for a second time in tap water, then placed in Schiffs reagent for 5-10 minutes. Next they were washed in tap water until they appeared pink-coloured. Finally, sections were dehydrated through different grades of ethanol, as described above, cleared in fresh xylene and mounted for cell analysis.

2.4.2.3 – Staining for Paneth cells (Fixed in 10% Formalin).

Sections were dewaxed in xylene then hydrated through graded alcohol to water, stained in Carazzi's haematoxylin for 10 minutes, then moved straight into 1% HCl in 70% alcohol for a few seconds. Next, they were washed in tap water, moved to phloxin stain and left for 20 minutes, rinsed in tap water, then blotted dry and moved to Tartrazine solution for not more than 10 minutes under microscopical observation. Sections were now rinsed in 95% alcohol and then twice in absolute ethanol. Finally, the sections were cleared and mounted in DPX.

2.4.2.4 – Staining for eosinophils (fixed in MT fixative)

Sections were deparaffinised and rehydrated through graded alcohols by dipping in each solution for no more than 1.5 minutes starting from xylene through to distilled water. Sections were then stained in 1% Astra blue (pH. 0.3) for 30 minutes, washed thoroughly in tap water until all surplus stain has been removed and then were transferred into Chromotrope 2R for another 30 minutes. Sections were briefly washed in tap water, quickly dehydrated through ascending grades of alcohol, cleared in xylene and mounted in DPX.

2.5 – ASSESSMENT OF INTESTINAL PATHOLOGY

2.5.1 – Villous/crypt measurements and quantification of mitotic figures.

Clark-fixed samples were placed in 10ml of 50% ethanol for 10 minutes then transferred into 20ml of tap water for another 10 minutes. Samples were then carefully placed in 10ml-preheated 1N HCl at 60°C for 7.5 minutes and

moved to 20ml of tap water for 10 minutes. Sections were washed three times in tap water until the piece of paper to which the tissues were originally attached had detached. Samples were placed in 2ml of Schiffs reagent (see appendix 2.8.2 -O-) for 20-30 minutes. Stained sections were washed in tap water allowing the extra stain to leave the section. Tissue muscularis layers were gently removed with fine forceps and the section was cut into four pieces under the dissecting microscope using a pair of fine forceps. Villi/crypts were cut into lines using Lang cataract knife (MSP, Cataract standard, super Sharps) and placed on microscope slide with a drop of 45% acetic acid and gently covered with a cover slip. Under a microscope fitted with a calibrated eyepiece graticule, sections were viewed and the lengths of 20 randomly chosen villi and crypts were measured. Next the slides were gently compressed with a pencil tip until the crypts had disrupted. The mitotic figures were differentiated and counted under a high power (X40).

2.5.2 – Mast and goblet cells counting

In the first few experiments (Experiment 1 and Experiment 3 in Chapter 3), a conventional cell counting approach was adopted. Cells were counted in every 20-villous/crypt unit of tissue and the results were expressed in terms of number of cells/VCU. For all the remaining experiments in chapter 3 (Experiments 2, 4, 5, 6 and 7) and those in all other chapters, a different technique was adopted.

The method was based on a Weible 2 graticule. The area covered by the Weible 2 graticule was first determined at each magnification at which it was used. This was achieved by using a calibrated micrometer slide to obtain lengths for the sides of the square encompassed by the Weible graticule. The 1mm slide graticule was used with 100, 0.01 mm divisions. With our microscope system the area covered by the Weible 2 graticule at X200 magnification was 1.172 mm^2.

The number of cells of interest was counted in terms of cells/mm^2 of mucosal tissue. In the following example, five cells are shown in the illustration (Fig 2.1) The Weible 2 graticule is marked with 21 lines, with interruptions as long as the lines themselves. The ends of these lines can be considered as distinct points, a total of 42 points. The number of these points, which lie over the tissue sections, was determined. In Fig 1, 18 of the 42 points lie on the tissue, giving a ratio of tissue to space within the section of 18/42. At X200 magnification, the area covered by the tissue is then: 18/42 X 1.172 mm^2 = 0.502 mm2. With five cells in this area, this is the equivalent of 9.960 cells/mm^{-2} (nearly 10 cell/mm^2).

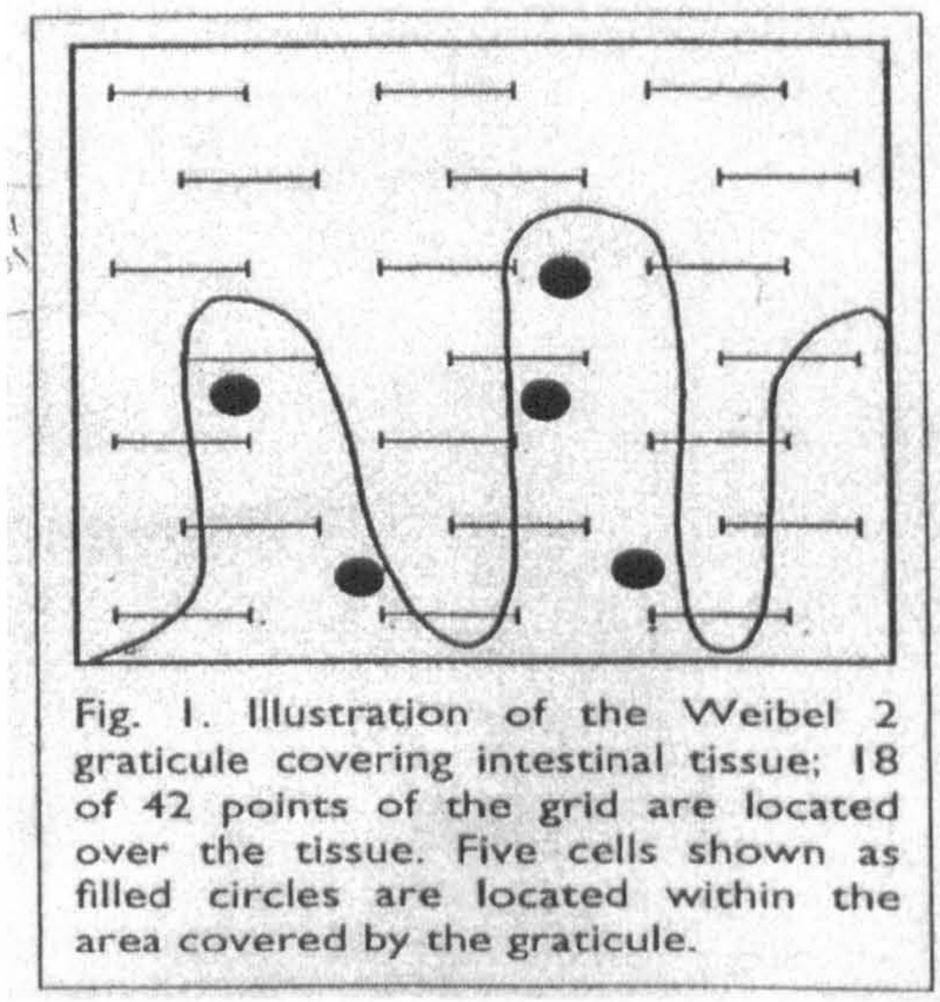


Fig. 1. Illustration of the Weibel 2 graticule covering intestinal tissue; 18 of 42 points of the grid are located over the tissue. Five cells shown as filled circles are located within the area covered by the graticule.

Figure 2.1 - Illustration of the layout box of Weible 2 graticule (Weibel, E.R, 1979)

2.5.3 – Paneth cells counting

Stained Paneth cells were counted in 20 crypts from each animal and the mean was calculated. Paneth cells that stained in contrasting colour patterns were counted separately, and if required a total cell counts was then calculated and the total was given.

2.5.4 – Haemagglutination Assay

Blood was collected at autopsy from the hamsters' hearts in experiment described in Chapter 6, into 1.5 Eppindorfs tubes, and was centrifuged at 1500-1800 rpm/5mins. Supernatant sera containing antibodies from each animal were serially diluted (in doubling dilutions) in physiological saline (PBS) and placed in wells (columns 1-10, read left to right) of the haemagglutination plate. Positive controls (column 11) and negative controls (columns 12) are included. A suspension of sheep red blood cells (TCS Biosciences Ltd, Botolph Claydon, Buckingham) and the reactions were observed. If sufficient antibody is present to agglutinate the cells, they sink as a mat to the bottom of the well. But, if insufficient antibody is present, the cells roll down the sloping sides of the plate to form a red pellet at the bottom.

2.6 – DRUG TREATMENTS

2.6.1 – Emtryl and Terramycin

Emtryl (Diametrdazole) was given in the drinking water at a concentration of 1g/L to all hamsters for 1 week prior to the start of each experiment, to prevent flagellate infections. Then in the following week, hamsters were treated with three grams of Terramycin (Pfizer, oxytetracycline hydrochloride) were dissolved in one Litre of tap water and given to hamsters daily for another seven days in order to minimize the risk of any enteric bacterial infection. Animals were left for another seven days on ordinary tap water to allow both drugs to clear.

2.6.2 – Ivermectin

Hamsters were individually weighed and given a recommended dose of Ivermectin as stipulated by the company ("Ivomec super" made in Holland for MSD AGVET, Division of Merk Sharp and Dohme Limited), which is (1ml/50kg body weight). Experimental work, using Ivermectin to terminate hookworm infection, indicated that this drug was effective in eliminating hookworm infections. Earlier work has shown that Ivermectin is an excellent drug for removing *Ancylostoma ceylanicum* (Behnke *et al.*, 1993; Richards *et al.*, 1995) As expected and reported in the previous articles, the dose used resulted in correlate clearance of the worms and therefore it used during the work of this thesis.

A stock concentration of 200µg/ml was prepared by a 1 in 50 dilution using distilled water. Each hamster was given 200µg/kg body weight of Ivermectin orally, which was calculated using the following formula:

200/1000 X weight of hamster = µg/hamster.

Since 1 ml contains 200 µg, the volume (mls) of Ivermectin to be administered = 1/200 X µg/hamster

2.6.3 – Cyclosporin A.

Cyclosporin A (Sandimmune, Sandoz, Pharmaceuticals, University of Nottingham, QMC), was supplied as 50mg/ml in caster oil and absolute ethanol and was diluted in PBS then administered daily for 9 days then every second day at a dose of 100 mg/kg of hamster's weight subcutaneously to hamsters in the experiment described in Chapter 6. This drug was used because of the well-known suppressive effect on T cells as reported Chapters 1 and 6 and because other methods of immunosuppression were not available for use in hamsters. However, there are no reports on treatment of animals infected with hookworms

with CsA, so the use of the drug in this context was novel and not entirely predictable.

2.7 – STATISTICAL ANALYSIS

The results are generally expressed as mean values ± the standard error of the mean (S.E.M.) for groups of animals that underwent similar treatment. Differences between groups were analysed using standard parametric one-way analysis of variance (ANOVA) or 2-way ANOVA as required. The latter was used particularly when time was one of the factors and treatment the other. If data did not confirm to normal distributions, the non-parametric Kruskal Wallis rank test (KR ANOVA) was used. Where appropriate correlations between variables were examined by the Spearman Rank Order correlation test and the r_s values are given. A probability $p< 0.05$ was considered to indicate a significant difference warranting rejection of the null hypothesis, and hence indicating no significant difference between groups. Statistical procedures were performed using the software package SPSS (V11.0).

2.8 – THE USE OF COMPUTER PROGRAMS

Some computer programs were used during the experimental work and writing of this thesis. These include the following (Microsoft Word processor 2000, which have been used to write this thesis in addition to other group or school reports; MS Excel 2000 spreadsheet for the production and presentation of the graphs and the calculation of the mean value throughout my study; MS PowerPoint 1997 & 2000 for the presentation of the results; Adobe Photoshop V-0.6 for the scan of the photos which taken from experimental samples under microscope to be presented on word documents; SPSS and StatGraphics for the analysis of the results and data; EndNote V-5 for references and bibliography) all were under the operating system of Windows 98 and lately XP.

CHAPTER THREE

MUCOSAL IMMUNE RESPONSES TO PRIMARY INFECTION WITH THE HOOKWORM *ANCYLOSTOMA CEYLANICUM*

3.1- SUMMARY

Seven experiments were carried out in female hamsters infected with either *A. ceylanicum* or *T. spiralis*, the later as a positive control. These included two preliminary experiments one of which (Experiment 1) compared cellular changes that occur in hamsters infected with each species. The other preliminary experiment (Experiment 2) was carried out to compare Paneth cell responses in hamsters and mice following infection with *A. ceylanicum* and *T. spiralis*. A further 5 experiments were carried out to measure, (1)-the time dependant changes in the cellular response of the mucosa to infection with *A. ceylanicum* (Experiments 3 & 4), (2)-dose dependant changes (Experiments 5 & 6) and (3)- recovery of the mucosa after removal of the worms by chemotherapy (Experiment 7). The results showed marked changes to the mucosal architecture – indicated by reduction in villous height and concurrent increase in crypt depth. As long as the adult worms remained in the intestine, the villi were shorter than normal and crypts deeper. These changes in the intestinal structure and inflammatory infiltrate were dependant on worm burden (Experiment 5 and 6), time-dependant (Experiment 3 and 4) and were also dependant on the presence of the worm (Experiment 7)

It is concluded, therefore, that following infection with *A.ceylanicum*, the intestinal mucosa of hamsters undergoes marked changes, reflected in both the mucosal architecture and in infiltration by inflammatory cells. These changes are dependent on time and intensity of infection and on the presence of worms. Differences in the rate at which changes occurred in an effector component of inflammation, such as mast cells, goblet cells and eosinophils suggest that to some degree different elements may be controlled independently, although centrally driven by the Th2 response.

3.2 – INTRODUCTION

Helminths, such as hookworms, still cause widespread infections among both people and our domestic animals, particularly in equatorial region of the world (Carroll *et al.*, 1988).

They are known to be particularly long-lived. The evidence for this comes from experiments in which volunteers have infected themselves. Two such experiments, both based on single-pulse infection with relatively small numbers of larvae, are particularly well known, one by Palmer (1955) and the other by Beaver (1988). The former found that the eggs of *N.americanus* were shed continuously for 17 years, whilst the latter found egg production to continue for 18 years. These chronic infections are believed to be facilitated by the evasive strategies employed by the worms (Behnke, 1987b; Ogilvie and Wilson, 1976; Pritchard, 1995a). Epidemiological evidence points to worms being continuously acquired with age (Behnke, 1987a; Pritchard, 1995b). In contrast to the age-intensity curves of many other helminths, hookworms typically show monotonic, asymptotic curves (Pritchard *et al.*, 1990; Quinnell *et al.*, 1993). Moreover, volunteer experiments in humans have shown that there is little acquired immunity (Ogilvie *et al.*, 1978). Nevertheless, some recent studies have indicated that immune responses may control worm fecundity in humans (Pritchard, 1995b). If the hookworms do indeed use evasive strategies that manipulate the local immune response, then we might expect to see some evidence of this at the mucosal level, perhaps reflected in changes (e.g. depression) in cells known to play a key role in host-protective immunity to other GI nematode infections.

The intestinal structure and the associated cellular immune responses of hosts infected with hookworms show changes which are attributable to infection and that are largely confined to the attachment sites of the worms in the mucosa and submucosa (Verma *et al.*, 1968). Dogs experimentally infected with moderate to heavy infection of *A. caninum* showed abnormal changes in villous height and crypt depth manifested by villous atrophy and crypt hyperplasia and an increase in Goblet cell activities (Migasena *et al.*, 1972a). The less pathogenic dog hookworm, *D. stenocephala*, causes focal damage to the gut that is essentially confined to the worm's feeding site (Gibbs, 1958).

Marked changes have also been reported in dogs experimentally infected with *A. ceylanicum* (Carroll *et al.*, 1984; Carroll *et al.*, 1985). This was expressed by severe villous atrophy, ulceration and infiltration by neutrophils and eosinophils in the areas surrounding the head of parasites embedded in the

lamina propria. However, changes that may occur during the course of infection have not been investigated thoroughly in the dog system because dogs are expensive and ethical considerations preclude large experimental groups.

Humans infected with hookworms show more than minor histopathological changes directly attributable to the presence of hookworms (Pimparker *et al.*, 1970). Intense infiltration of the mucosa with inflammatory cells concentrated at the parasite's feeding site has been reported in hospitalised patients during hookworm infection. These were illustrated in the work carried out by Chaudhuri and Saha (1964) who reported an increase in goblet cells and focal eosinophilic accumulation but no substantial damage to the mucosa. In the single case of marked eosinophilic accumulation in the submucosa, the biopsy specimen probably corresponded to a feeding site. Moderate changes were found by Burman (1970) in the jejunal mucosa in 4/30 patients with *A. duodenale* whereas (Nath *et al.*, 1971) studying a group of 60 subjects patients detected moderate changes in only 11 out of 60 with 2 additional cases of extreme abnormalities. Marked intestinal changes were reported during severe *A. duodenale* infection in adults (Salem and Truelove, 1964) and children (Zimmerman, 1946). Rai *et al.* (1968) suggested that severity of the mucosal changes might be attributable to the intensity of the infection. However, this view contrasts with most other studies, such as those by Aziz and Siddiiqui (1968) who concluded that morphological abnormalities of the intestinal mucosa bear little relationship to the intensity of hookworm infection.

Experiments with hamsters infected with *A. ceylanicum* revealed also marked mastocytosis and goblet cell hyperplasia during the course of a single pulse primary infection (Garside and Behnke, 1989). Furthermore, data from Behnke (1991) showed that these changes were accompanied by villous atrophy, where the viili changed from a long and slenderic shape to a club-shaped morphology and crypts deepened. These changes are all consistent with the involvement of cellular mucosal responses. Nevertheless, despite the intensity of these reactions, the worms survive for many weeks after their onset, seemingly unaffected by the mucosal changes.

Following on from Behnke *et al.* (1997), the current chapter explores changes that occur during a single-pulse primary infection with *A. ceylanicum* in hamsters. In addition, to monitoring mucosal mast cell numbers, as was done in earlier studies (Behnke and Rose, 1997), other components of the Th-2 driven mucosal immune response were also quantified i.e. goblet cells, Paneth cells and eosinophils. Moreover, the architectural changes in the surface of the mucosa were studied through quantification of changes in villous height and Crypt of Lieberkuhn depth, and in the cellular division in the enterocyte population.

3.3- EXPERIMENTAL DESIGN AND RESULTS

Seven experiments were carried out in female hamsters infected with either *A. ceylanicum* or *T. spiralis*, the latter as a positive control since it is known to provoke an immune response in this susceptible host. These included two preliminary experiments one of which (Experiment 1) compared cellular changes that occur in hamsters infected with each species. The other preliminary (Experiment 2) was carried out to compare Paneth cell responses in hamsters and mice following infection with *A. ceylanicum* and *T. spiralis*. A further 5 experiments were carried out to measure, (1)-the time dependant changes in the cellular response of the mucosa to infection with *A. ceylanicum*, (2)-dose dependant changes and (3)- recovery of the mucosa after removal of the worms by chemotherapy

3.3.1 – Preliminary experiments

As can be seen from Table 3.1, the first Experiment consisted of a group of four hamsters with no infection and these provided information on background concentration of cells in the mucosa of uninfected animals. Two additional groups of four hamsters received 500 Larvae of *T. spiralis* and were killed on day 10 post infection (pi) for both assessments of mucosal changes

Table 3.1 – Experiment 1. (Preliminary Experiment). Experimental design to investigate the influence of *A.ceylanicum* and *T. spiralis* on host cellular responses and the architecture of the small intestine.

No. of hamsters	Dose of L3 given	Mean no. of worms recovered	Use
4	Nil	0	Histology*
4	200 *A. ceylanicum*	83.5 ± 8.341	Histology* + worm counts
4	200 *A. ceylanicum*	101.5 ± 15.069	Worm counts
4	500 *T. spiralis*	91.5 ± 7.170	Histology* + worm counts
4	500 *T. spiralis*	125.5 ± 13.143	Worm counts

* Parameters measured at autopsy were:

- Villous height & crypt depth. (Fig 3.5 – A)
- Mitotic figures. (Fig 3.5 – B)
- Mast cells. (Fig 3.6)
- Goblet cells (Fig 3.7)

and worm count. Another two groups of four hamsters were infected with 200 L3 *A. ceylanicum* and then killed at the same time as the previous group.

Table 3.2 shows the design of Experiment 2, which was carried out to compare the Paneth cell responses in mice and hamsters. This shows how the total number of 30 female DSN hamsters and 30 NIH mice were allocated to groups of five and divided into three different treatments. The first category consisted of two groups of five animals from each host, which were left as uninfected control groups. The second category included two more groups of five which received an infection with 100 L3 *A. ceylanicum* and finally, the last two groups were infected with 200 L3 *T. spiralis*. One group from each category was killed on day 12 and the other on day 22 pi. Worms were collected and counted and Paneth cell number were assessed. Animals from both experiments were weighed carefully once a week before the infection and twice a week following infection.

3.3.1.1 – Changes in the weight of hamsters

Fig 3.1 shows changes in the weight of hamsters in four of the infected groups and the uninfected control group before and after infection (Experiment 1). All five groups gained weight before infection. However, the four infected groups started to lose weight gradually from day 6 after infection. A sharper reduction in the weight was noticed on day 10, which is the day of which all groups were culled. From the illustrated data, it can be deduce that worms had established within the hamsters' intestines and started obtaining their nutrients.

Similarly, Figures 3.2 illustrates the weights of hamsters and mice (A and B respectively) in Experiment 2. It was noticed that there was a steady increase in all groups of animals during the course of this experiment before they became infected and then reduction or stability in the weight of infected groups from both hosts was recorded a few days after infection, which suggested that worms had established in the intestines.

Table 3.2 – Experiment 2. (Preliminary Experiment) Experimental design to compare Paneth cell responses in hamsters and mice infected with *A.ceylanicum* and *T. spiralis*.

No. of animals used	Dose of larvae given	Mean No. of worms recovered	Day killed
5 hamster	Nil	Nil	12
5 hamsters	Nil	Nil	22
5 hamsters	200 *T. spiralis*	41.20 ± 4.15	12
5 hamsters	200 *T. spiralis*	53.40 ± 8.53	22
5 hamsters	100 *A. ceylanicum*	19.60 ± 2.84	12
5 hamsters	100 *A. ceylanicum*	14.20 ± 1.66	22
5 mice	Nil	Nil	12
5 mice	Nil	Nil	22
5 mice	200 *T. spiralis*	20.40 ± 2.66	12
5 mice	200 *T. spiralis*	27.80 ± 2.82	22
5 mice	100 *A. ceylanicum*	0	12
5 mice	100 *A. ceylanicum*	0	22

Note: Only Paneth cells were counted at autopsy in this experiment in order to compare the response between two different hosts infected with two different nematode species.

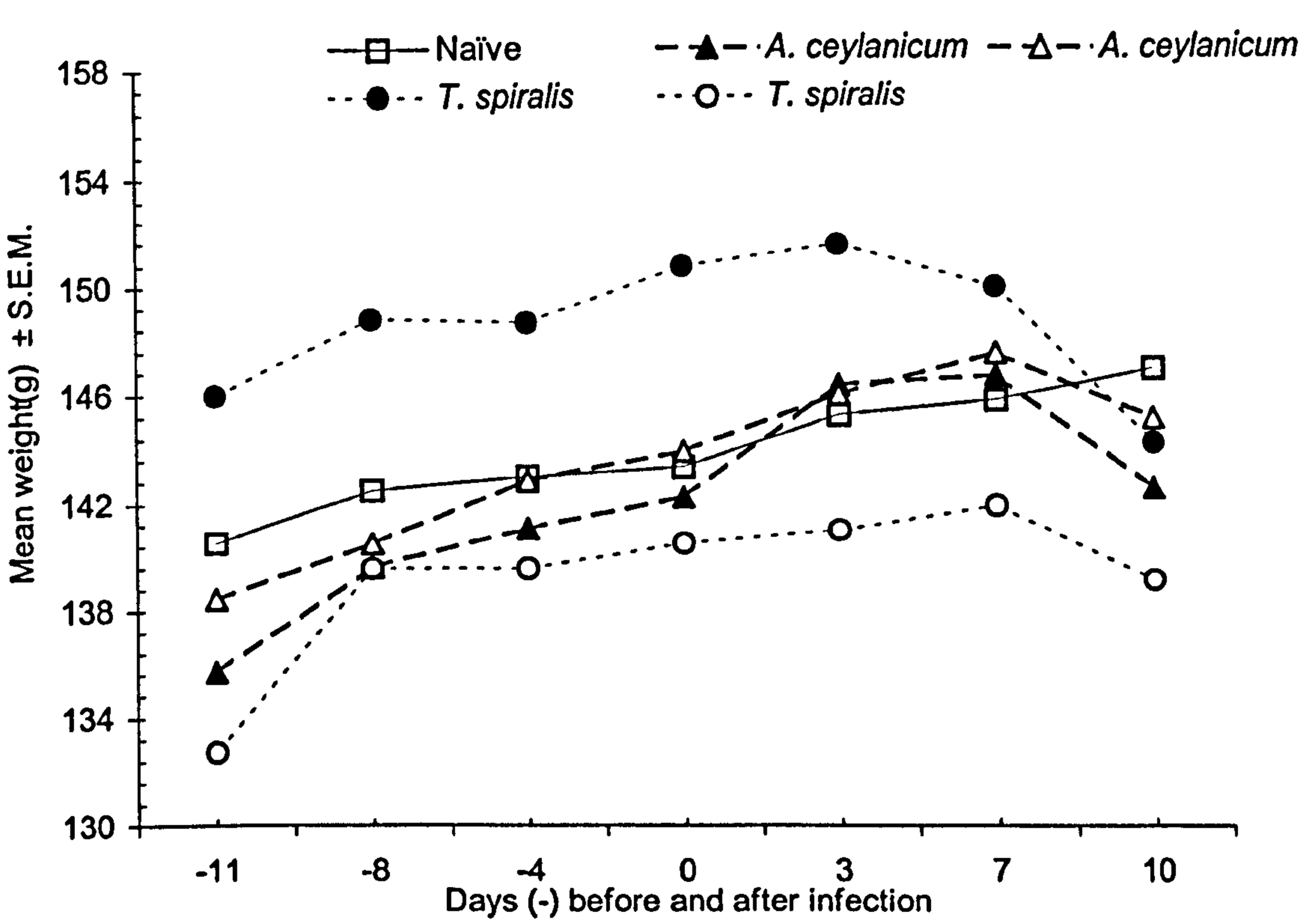
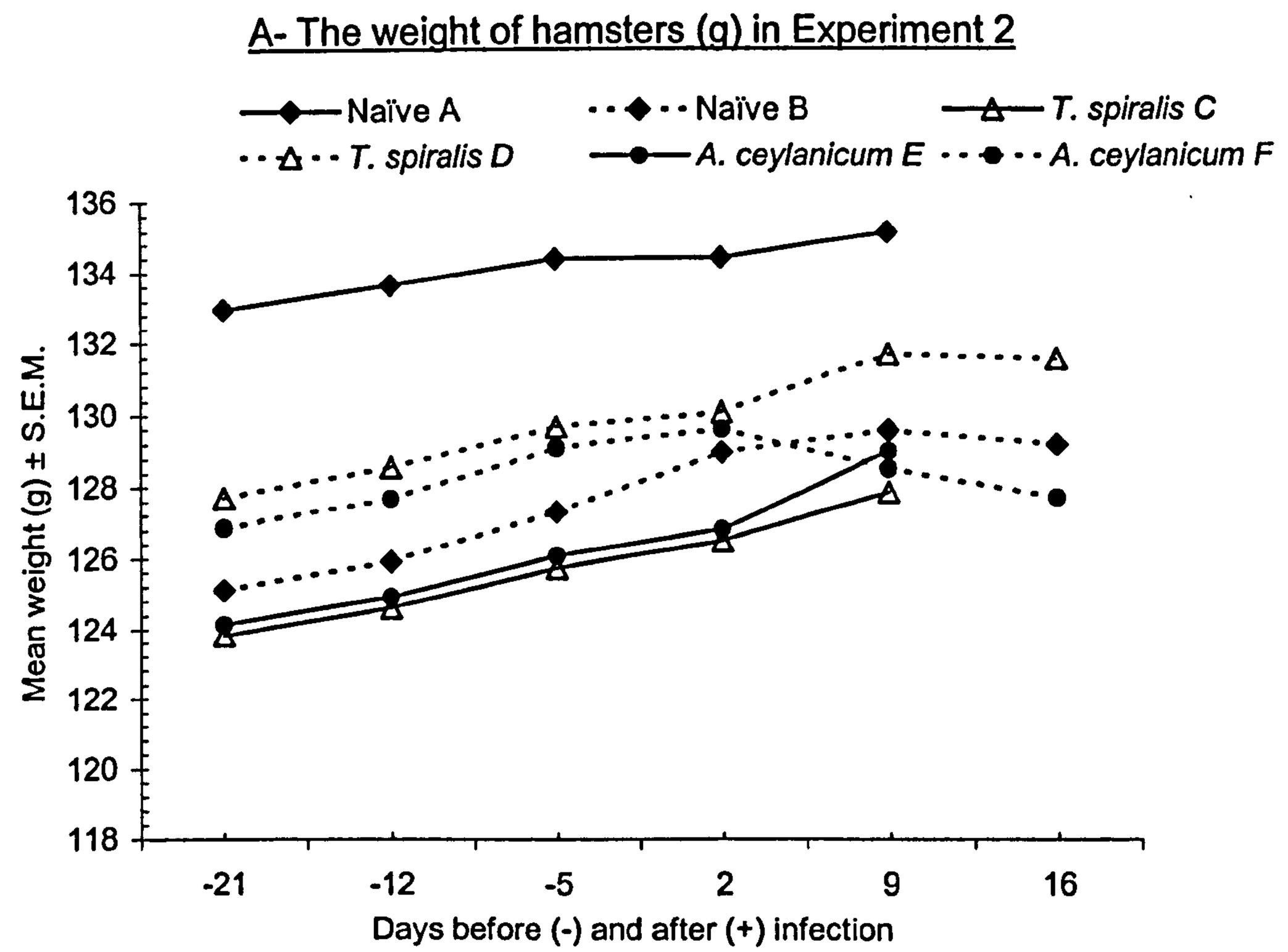


Figure 3.1 Experiment 1. (Preliminary Experiment). The weight of hamsters before (-) and after (+) infection. Control ( ) group of hamsters, (and Δ) two groups infected with 200 L3 *Ancylostoma ceylanicum* and (and o) 2 groups of hamsters infected with 500 Larvae of *Trichinella spiralis*



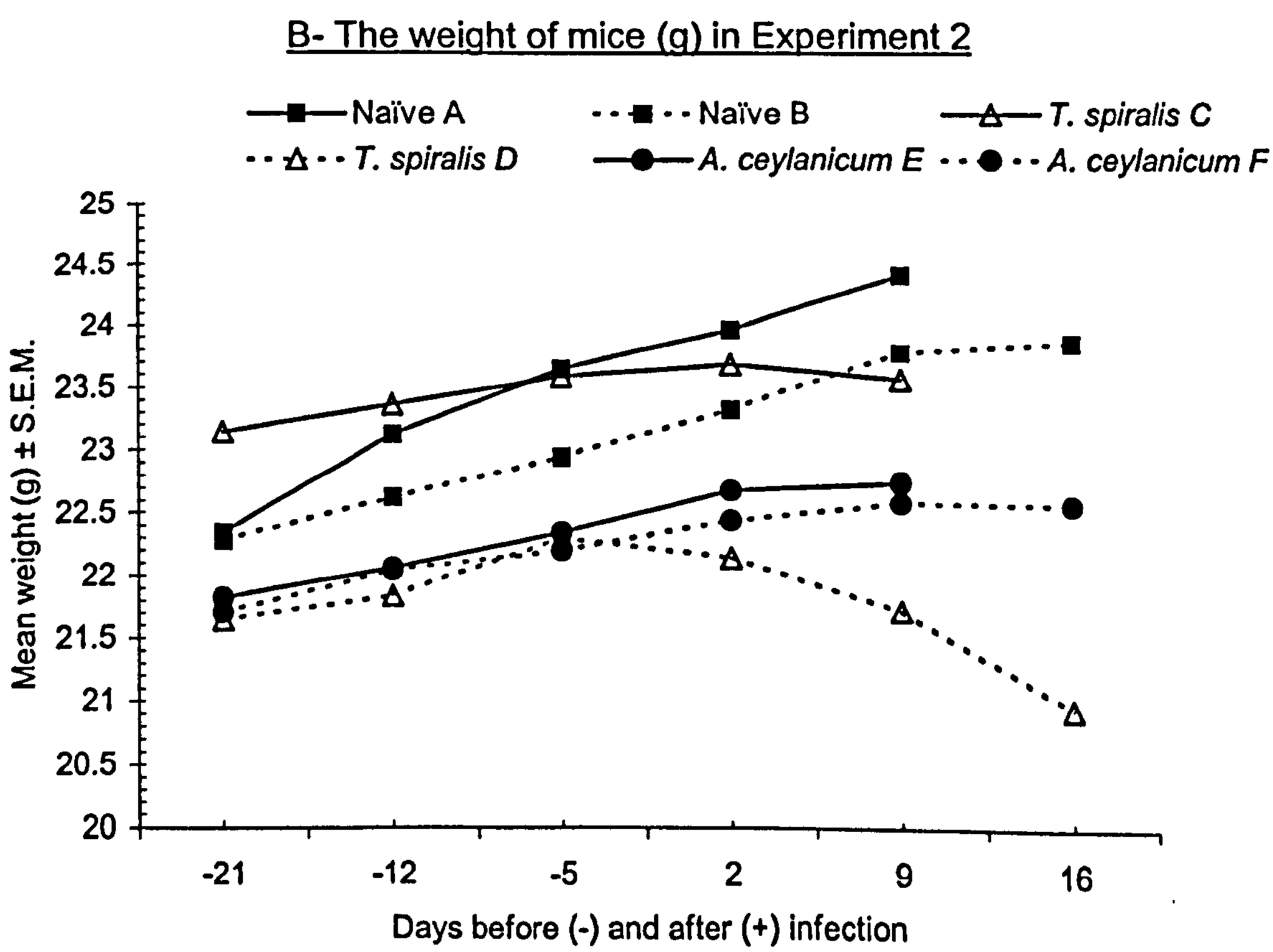


Figure 3.2 Experiment 2. (Preliminary Experiment). The weight of animals before (-) and after (+) infection. Hamster weights (A) and mouse weights (B).

3.3.1.2 – Worm recovery

Figure 3.3 and Figure 3.4 summarise the mean number of worms (*A. ceylanicum* and/or *T. spiralis*) recovered form the intestines of animals used in experiments 1 and 2 respectively.

3.3.1.3 – Assessment of Intestinal pathology (Exp. 1)

Mucosal architecture and epithelial cell mitotic activity were measured in the intestinal samples of Experiment 1 as described in the previous chapter (Chapter 2). Ten days after infection, intestinal pathology as indicated by villous atrophy, crypt hyperplasia and the increase in mitotic activity was quantified in the three groups indicated in Table 3.1 The results illustrated in Fig 3.5 - A, are shown as mean value ± S.E.M. for each group. Villous lengths were significantly reduced compared to the uninfected control group on day 10 pi (H=8.192, n=5,5,5 P<0.05). Hamsters infected with *T. spiralis* showed more marked changes in respect of the atrophy in villous length compared to the other group infected with *A. ceylanicum*. Crypt depth and changes in the cell division (mitotic fig) illustrated in Fig 3.5-B were significantly greater in infected compared with control animals (H=8.769, n=5,5,5 P=<0.05 and H=9.718, n=5,5,5 P=<0.01 respectively).

3.3.1.4 – Mast cell responses (Exp. 1)

Mast cells in Experiment 1 were quantified and the data are summarized in Fig 3.6. There was a significant increase in mast cells counts in both infected groups of hamsters. Increases were from approximately 30.58/20 V.C.U. in uninfected hamsters up to 87.84 cells/20 V.C.U. in the *Ancylostoma ceylanicum* infection and 72 cells/20 V.C.U. in *T. spiralis* infection (H=8.346, n=4,4,4, P=<0.05).

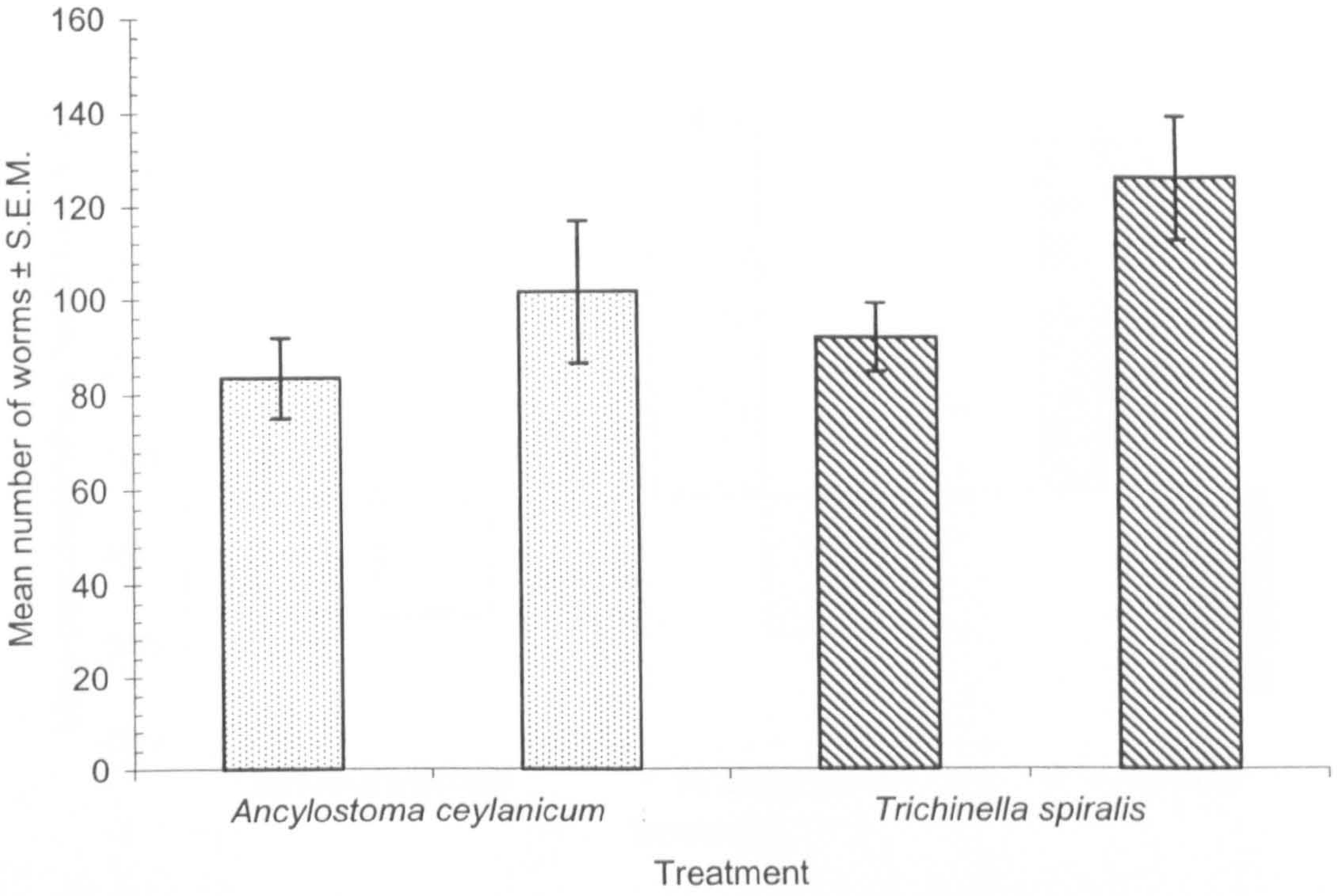


Figure 3.3 – Experiment 1 (Preliminary Experiment). Mean number of worms (*A. ceylanicum* ⬚ and *T. spiralis* ▨) recovered on day 10 pi.

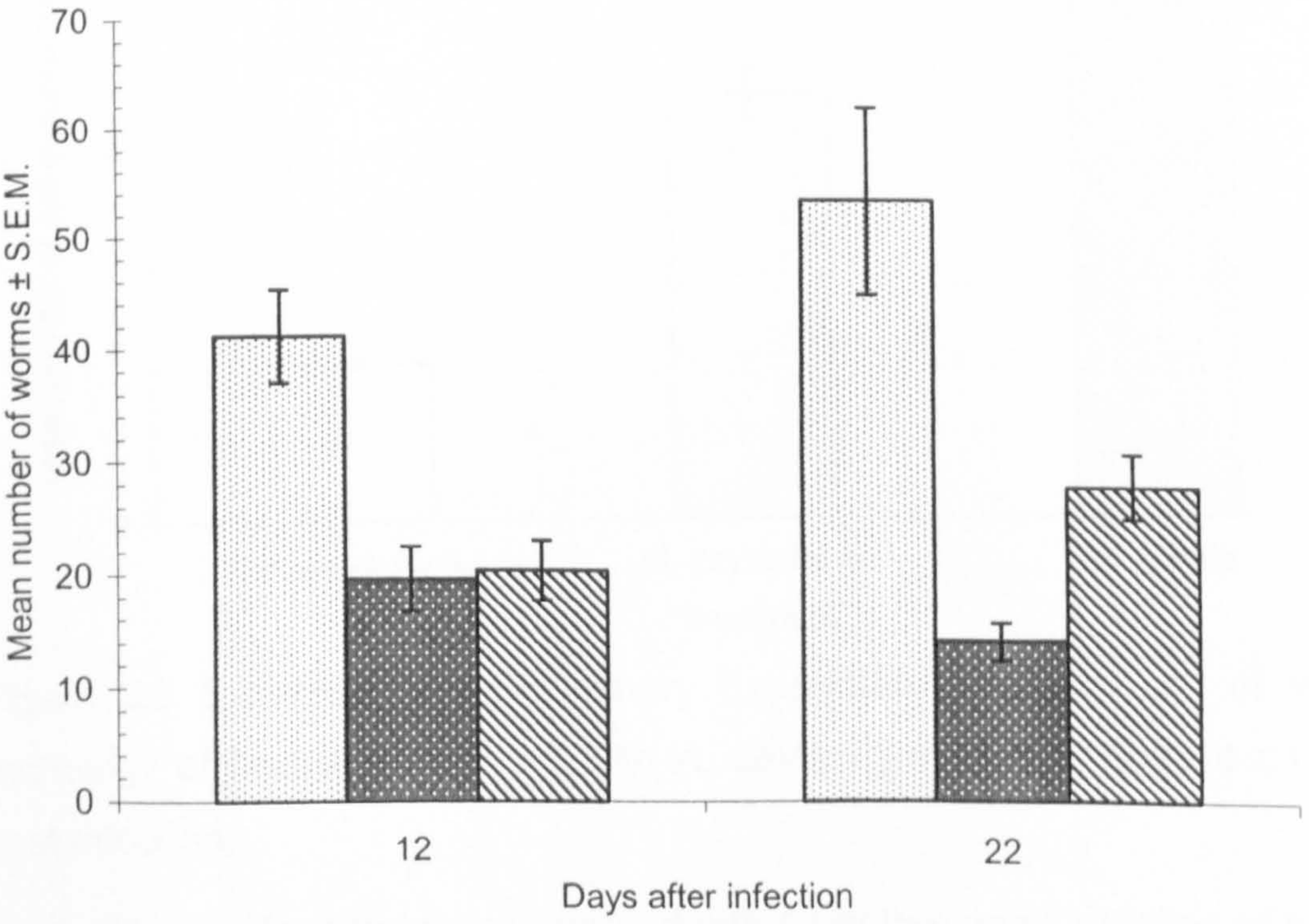


Figure 3.4 – Experiment 2 (Preliminary Experiment). Mean number of worms (*A. ceylanicum* in hamsters ⬚, *T. spiralis* in hamsters ▩ and in mice ▨) recovered on days 12 and 22 pi.

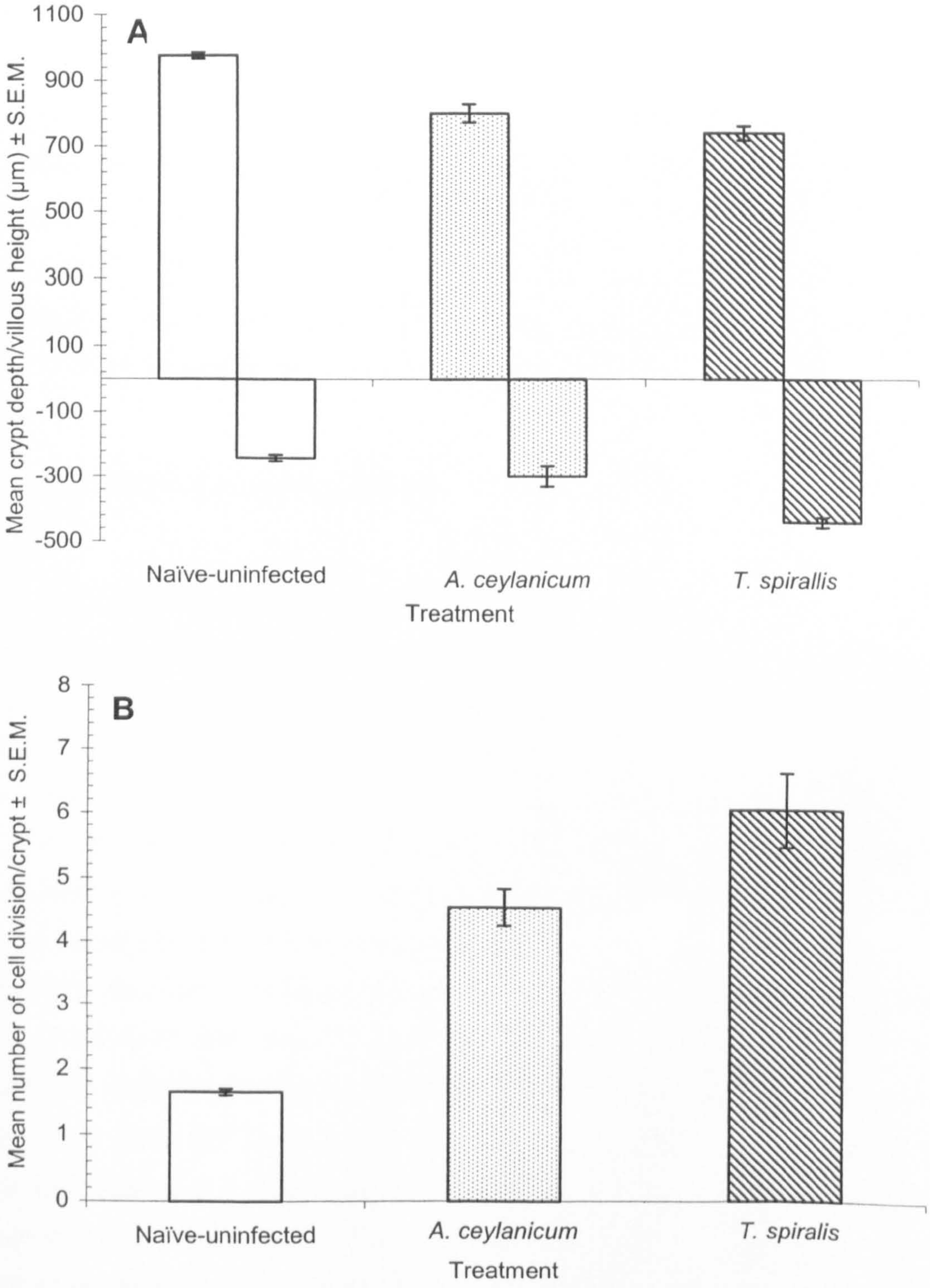


Figure 3.5 Experiment 1 (Preliminary Experiment). Assessment of intestinal pathology of hamsters infected with *A. ceylanicum* and *T. spiralis* on day 10 post infection.

A- Mean villous height (+)/ crypt depth (-) in the small intestine of hamsters killed on day 10 pi for three different treatment.

B- Mean number of mitotic figures per Villous/crypt unit

3.3.1.5 – Goblet cell responses (Exp. 1)

Quantification of changes in goblet cell numbers in the intestine of hamsters was carried out similarly to mast cells and is illustrated in Fig 3.7. It was found that goblet cells increased significantly from 126.84 cells/ 20 V.C.U. in the control group up to 284.86 cells/20 V.C.U. in *A. ceylanicum* infected hamsters to a peak count in excess of 335.5 cells/ 20 V.C.U. in hamsters infected with *T. spiralis* (H=7.511, n=5,5,5 P=<0.05).

3.3.1.6 – Paneth cell responses (Exp. 2)

Comparative data for Paneth cell numbers in the crypts of the intestines of hamsters and mice infected with *A. ceylanicum* and *T. spiralis* are illustrated in Figure 3.8. These show that the Paneth cell response of mice and hamsters differed even when infected with the same species of parasite. Analysis by 3-way ANOVA showed no significant main effect of treatment on Paneth cells ($F_{2.48}$=2.980, P=0.060) or time ($F_{1.48}$=2.893, P=0.095). However, Paneth cells were significantly affected by host species ($F_{1.48}$=373.361, P<0.001) and there was a highly significant interaction between host and treatment ($F_{2.48}$=21.711, P<0.001), time and treatment ($F_{2.48}$=8.093, P<0.001) but no 3-way interaction (host, treatment and time, $F_{2.48}$=0.608, P=0.549). By day 12, there was a significant reduction in Paneth cell counts of both infected groups compared to naïve hamsters. On day 12 post infection (pi), Paneth cell numbers fell from approximately of 1.3 cells/crypt in naïve uninfected hamsters to less than 1 cell/crypts in *T. spiralis* infected animals and 1.04 cells/crypt in hamsters with *A. ceylanicum* infection. A similar reduction from 2.6 cells/crypts in naïve hamsters to 1.15 cells/crypts in *T. spiralis* infected hamsters and 0.64 in *A. ceylanicum* infection was seen on day 22. However, on day 22 the reduction in Paneth cell numbers in hamsters appeared to be greater in animals given *A. ceylanicum* compared with those given *T. spiralis*, contrasting with the data on day 12 when the reduction was greater in the animals given *T. spiralis*.

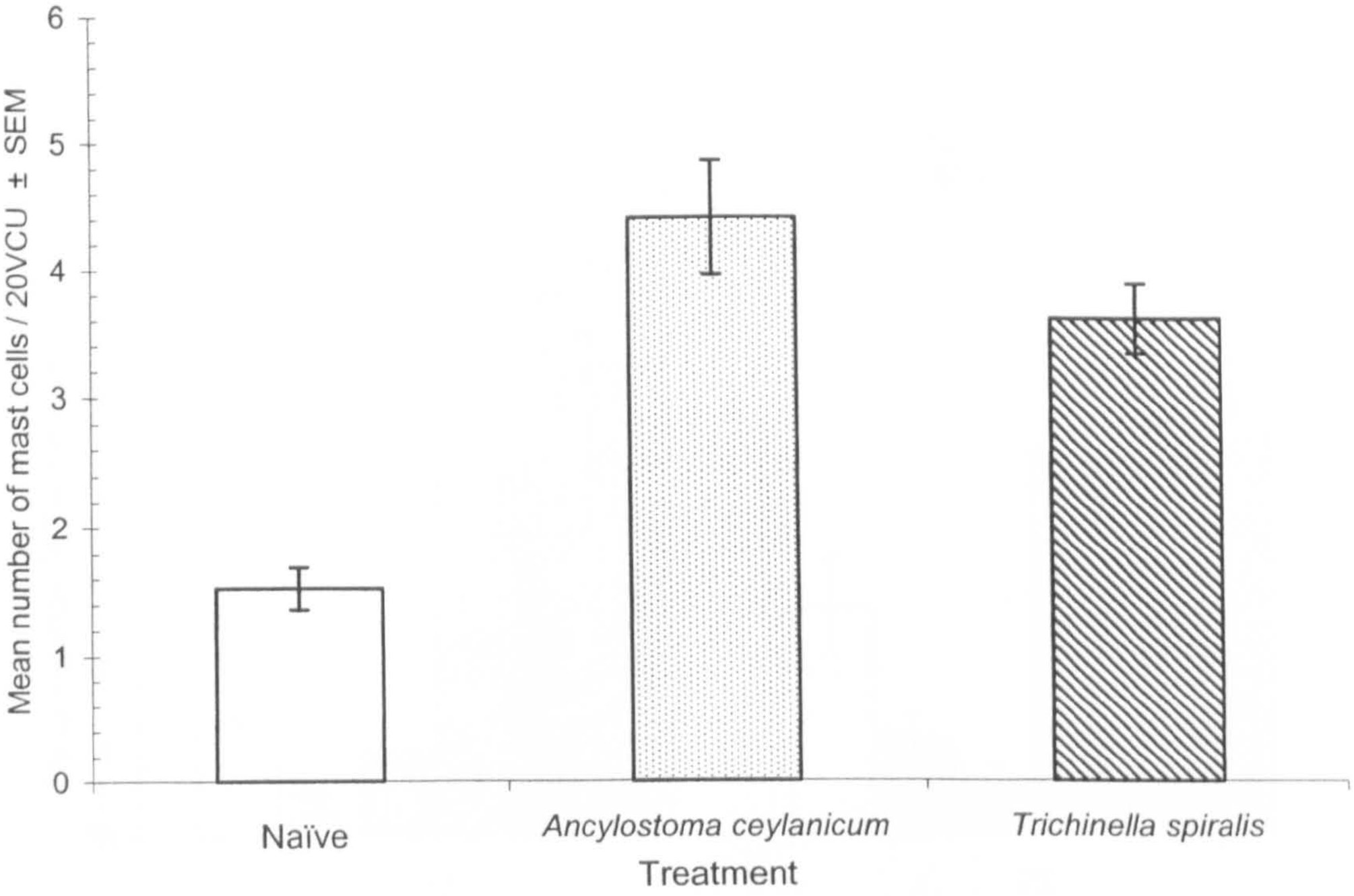


Figure 3.6 – Experiment 1- (Preliminary experiment). Mean number of mast cells /20 V.C.U. of the small intestine in hamsters ± SEM (day 10 after infection)

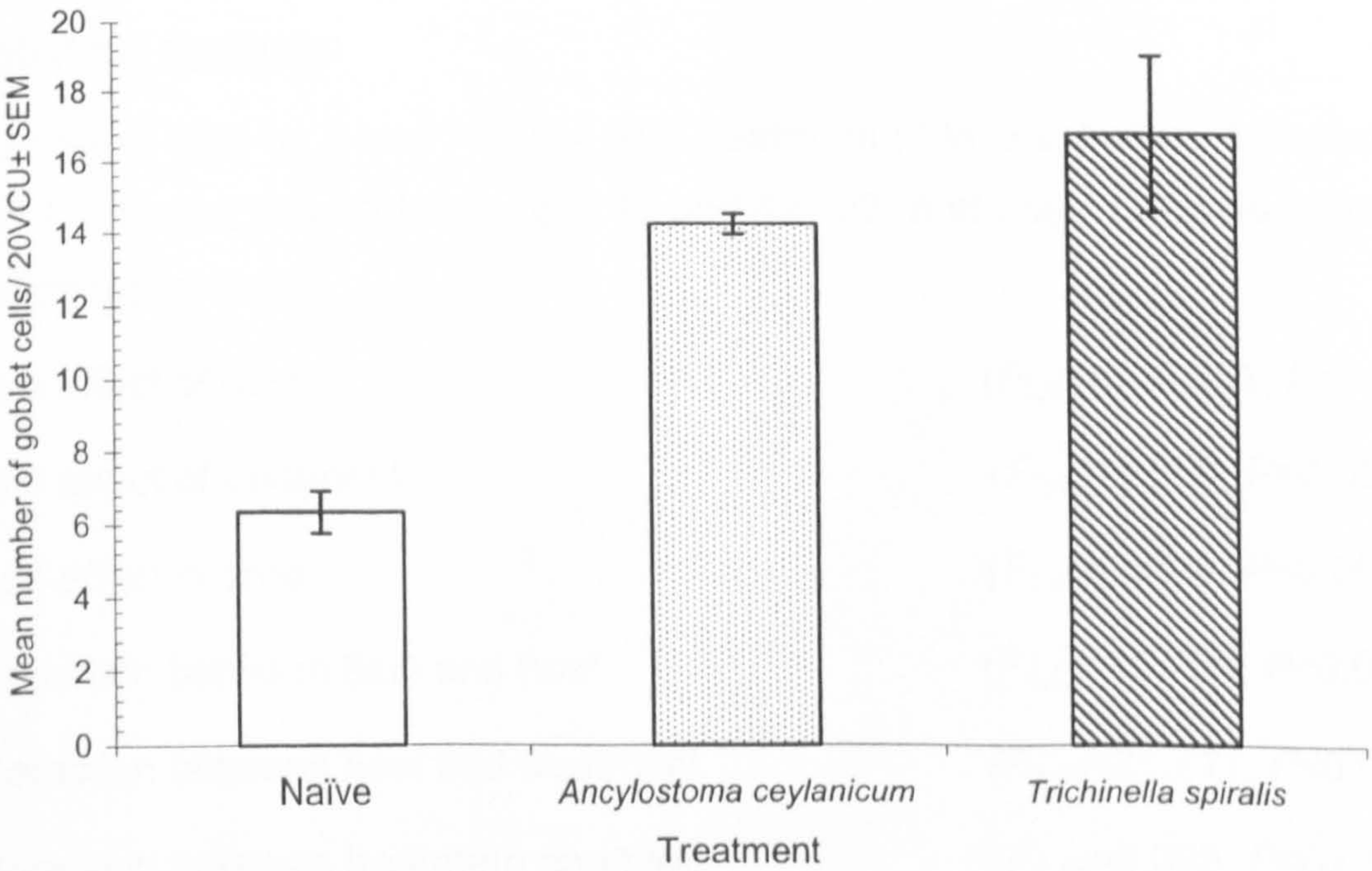


Figure 3.7 – Experiment 1 (Preliminary experiment). Mean number of goblet cells/20 V.C.U. ± S.E.M. in the small intestine of hamsters with three different treatments (Naive, *A. ceylanicum* and *T. spiralis* and killed day 10 after infection

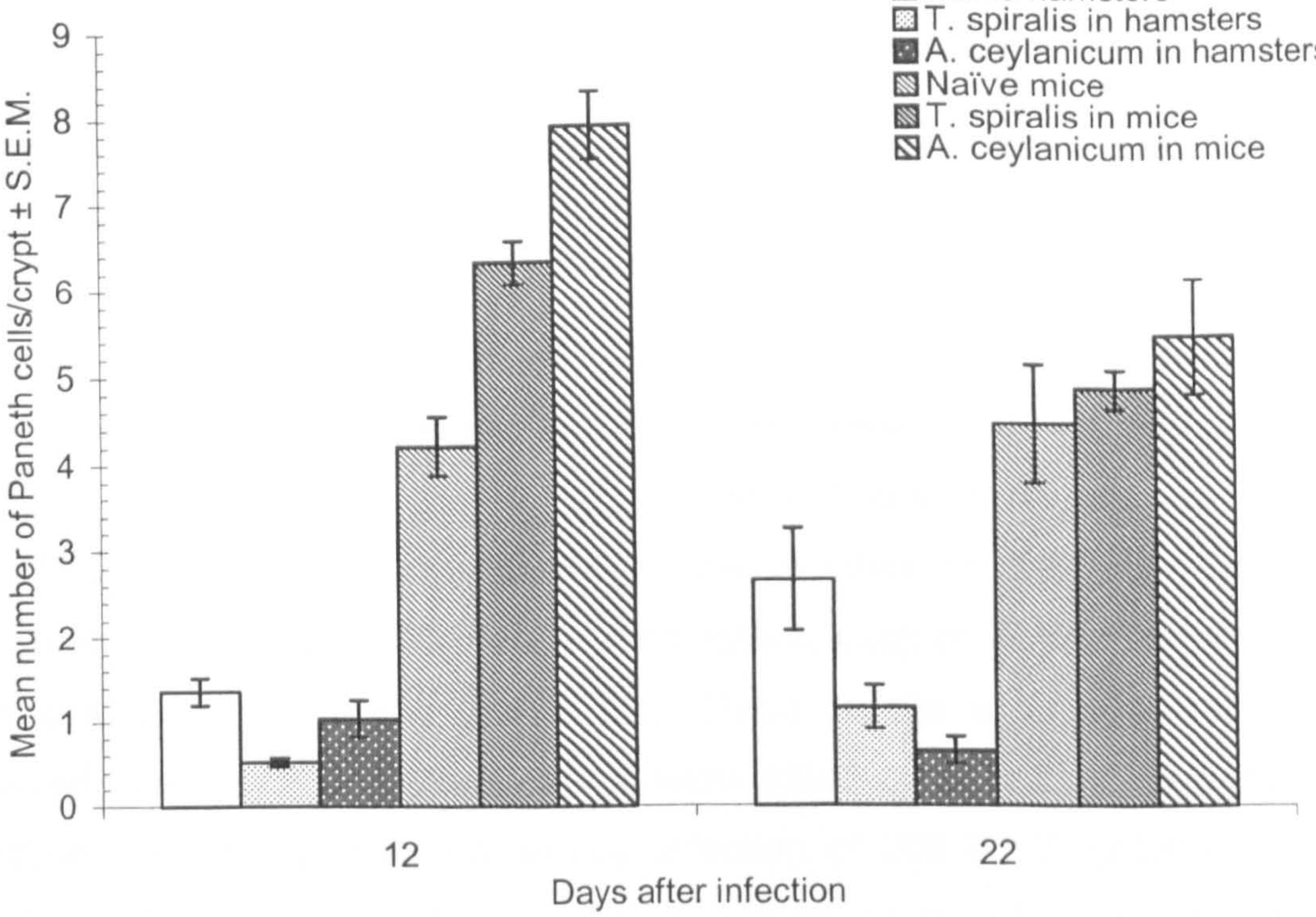


Figure 3.8 – Experiment 2 (Preliminary experiment). Comparison between the mean number of Paneth cells/crypt in the intestine of hamsters and mice infected with *A. ceylanicum* and *T. spiralis* on day 12 and 22 post infection.

Statistical analysis:

Analysis of data by 3-way ANOVA with treatment (3 levels, Naïve, *A.ceylanicum* and *T. spiralis*), time (2 levels, day 12 and day 22) and hosts (2 levels, hamsters and mice) revealed:

Main effect of host	($F_{1,48}$=373.361, P<0.001)
Main effect of treatment	($F_{2,48}$=2.980, P=0.060)
Main effect of time	($F_{1,48}$=2.893, P=0.095)
Interaction between time and host	($F_{1,48}$=15.709, P<0.001)
Interaction between host and treatment	($F_{2,48}$=21.711, P<0.001)
Interaction between treatment and time	($F_{2,48}$=8.093, P<0.01)
Interactions between treatment, time and hosts	($F_{2,48}$=0.608, P=0.549)

In mice, Paneth cell numbers rose following infection and the increase appeared to be more marked in animals with *A. ceylanicum*.

3.3.2 – Time-course experiment

To investigate the effect of infection with hookworms on intestinal cellular immune response over a longer period of time, two experiments were carried out. These experiments (3 and 4) were initiated at two different times. Experiment 3 comprised 10 groups of hamsters, each of which contain either 4 or 5 aged-match hamsters (Table 3.3). Three groups were not infected and received water only and food. They were killed on day 0, 7 and 42 after infection. One group received an oral infection of 200 L3 *T. spiralis* and was killed on day 14. The six remaining groups were infected with 100 L3 *A.ceylanicum* intragastrically and a group from this final treatment was killed every 7 days after infection starting from day 7 after primary infection.

Experiment 4 was carried out similarly but on a different occasion. A different way of measuring mast and goblet cells was introduced, using a Weible graticule. A group of 5 hamsters received a single dose of 200 L3 *T. spiralis* (group K) and they were killed on the same day after infection as in the previous experiment. Groups A, B and D received water only and were killed on days 7, 14 and 42 respectively. All the other remaining groups received a single dose of 100 L3 *A. ceylanicum* and were killed on a weekly basis starting 7 days after initial infection (Table 3.4 Experiment 4)

3.3.2.1 – Worm recovery

Changes in parasite burdens with time after infection for experiments 3 and 4 are illustrated in Fig 3.9 (A and B respectively). Worm burdens in both experiments vary between groups. One-way ANOVA (SPSS package) showed that there were significant differences between numbers of worms recovered from infected hamsters, on different days after infection in experiment 3

Table 3.3 – Experiment 3. (Time-course). The experimental design to investigate the effect of time on the parameters of the mucosal cellular immunity and the intestine architecture in hamster intestine during *A. ceylanicum* infection.

No. of hamsters used	Dose of L3 given	Mean no. of worms recovered	Day killed
5	Nil	Nil	0
5	Nil	Nil	7
5*	Nil	Nil	42
5	100 *A. ceylanicum*	39.2 ± 3.878	7
5	100 *A. ceylanicum*	35.4 ± 1.860	14
5	100 *A. ceylanicum*	32.6 ± 3.009	21
4	100 *A. ceylanicum*	30.75 ± 4.607	28
4	100 *A. ceylanicum*	27 ± 0.557	35
5*	100 *A. ceylanicum*	15.25 ± 6.523	42
4	200** *T. spiralis*	54.25 ± 6.074	14

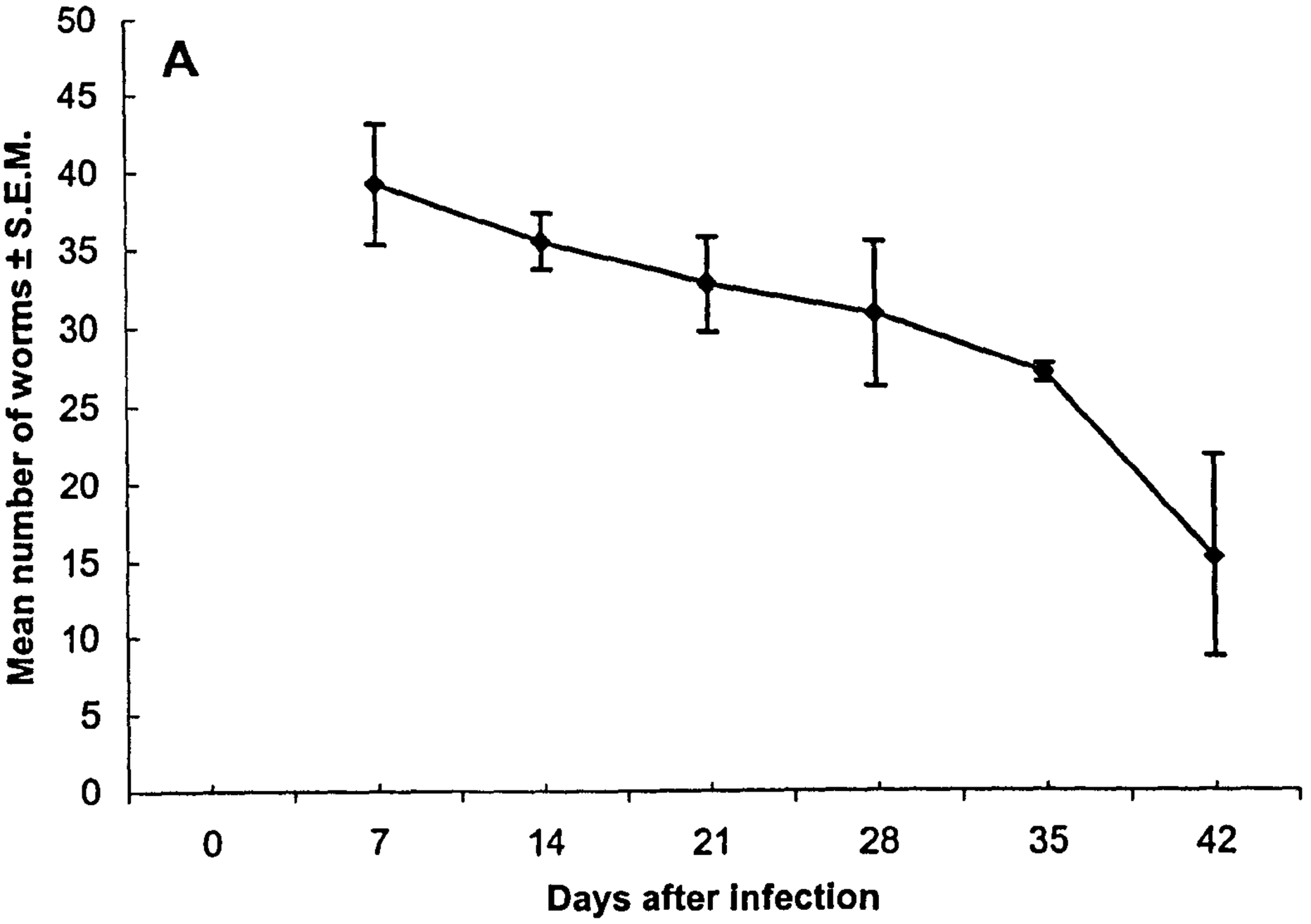
* One hamster died before the end of the experiment.

** This dose is known to induce a good immune response in hamsters by day 14 and was used as a positive control.

Table 3.4 – Experiment 4 (Time course). The experimental design to investigate the effect of time on the parameters of the mucosal cellular immunity and the intestine architecture in hamster intestine during *A. ceylanicum* infection.

No. of hamsters	Dose of L3 given	Mean no. of worms recovered	Day killed
5	NIL	NIL	0
5	NIL	NIL	7
5	NIL	NIL	42
5	100 *A. ceylanicum*	12.2 ± 0.969	7
5	100 *A. ceylanicum*	19 ± 4.099	14
5	100 *A. ceylanicum*	9.8 ± 1.241	21
5	100 *A. ceylanicum*	9 ± 3.507	28
5	100 *A. ceylanicum*	21 ± 3.808	35
5*	100 *A. ceylanicum*	12.25 ± 2.136	42
5	200 *T. spiralis*	13.4 ± 3.458	14

* One hamster died before the end of the experiment.



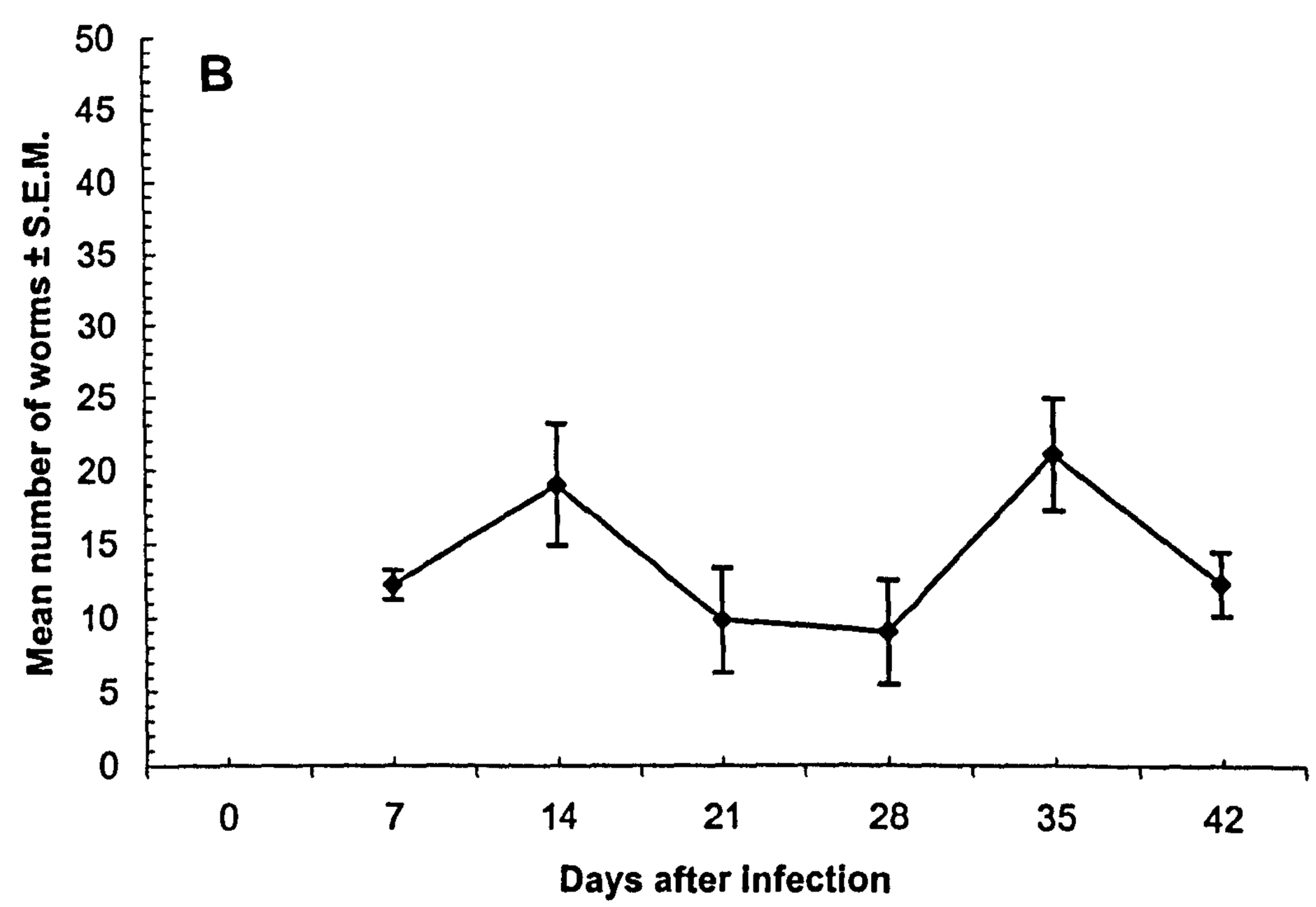


Figure 3.9 – Recovery of *A. ceylanicum* from time-course experiments

(A) Experiment 3 (Time-course) from day 7 to day 42 after infection.

(B) Experiment 4 (Time-course) from day 7 to day 42 after infection.

($F_{5,20}$=4.505, P<0.05). A weaker significant difference between groups in experiment 4 can be seen ($F_{5,23}$=2.881, P=0.037), arising principally from the relatively heavier parasites burdens on days 14 and 35. Changes of worm burden with time were analysed by Spearman rank order test and for experiment 2, analysis was significant (r_s=-0.619, n=26, P<0.01) whereas in experiment 3, this test showed a non-significant relationship between time and number of worms recovered (r_s=-0.098, n=29, P=0.611).

3.3.2.2 – Assessment of intestinal pathology

There were pronounced changes in the height of villi and the depth of the crypts of Lieberkuhn during infection in both Experiments 3 and 4 (Fig. 3.10 - A & B). Analysis by 2-way ANOVA of data from both experiments with infection and time as factors showed a significant main effect of time on the length of villi ($F_{5,27}$=55.225, P<0.001 and $F_{6,36}$=212.290, P<0.001, Experiments 3 and 4 respectively) but more importantly a significant interaction between treatment and time ($F_{1,27}$=10.078, P<0.01 and $F_{1,36}$=121.590, P<0.001). As can be seen in naïve hamsters in both experiments the mean height of villi was relatively constant over the course of these experiment (experiment 3 –range 772.625 µm ± 46.694 on day 0 after infection and 1010.625 µm ± 13.593 on day 42, Experiment 3 – range from 1060 µm ± 13.181 on day 0, 1071 µm ± 7.730 on day 14 and 1093.5 µm ± 12.031 on day 42) although the slightly lower value on day 0 in Experiment 3 suggests that despite precautions, animals may have suffered from some additional enteric infection. Nevertheless, villous height in infected hamsters declined markedly with time in both experiments with a reduction from 876 µm ± 23.974 on day 7 to 371.875 µm ± 24.354 on day 28 in Experiment 3 and from 959.5 µm ± 4.704 on day 7 to 178.5 µm ± 17.653 on day 35 in Experiment 4. However, both experiments showed some indication of a partial increase towards the end (186.5 µm ± on day 42 post infection in Experiment 4 and 407.5 µm ± 6.292 on same day of Experiment 3.

There were similar marked differences between infected and control animals in changes in the depth of crypts with time. The depth of the crypt

Figures 3.10 - Mean villous height (+) and crypt depth (-) (μm) in the intestine of hamsters infected with *A. ceylanicum*, *T. spiralis* and neither treatment from day 0 to day 42.

(A) Experiment 3 (Time-course experiment).

(B) Experiment 4 (Time-course experiment).

Statistical analysis:

Data analysis from Experiment 3 by 2-way ANOVA with time (6 levels, 0, 7, 14, 21, 28 and 42) and treatment (2 level, Naïve and *A. ceylanicum*) showed:

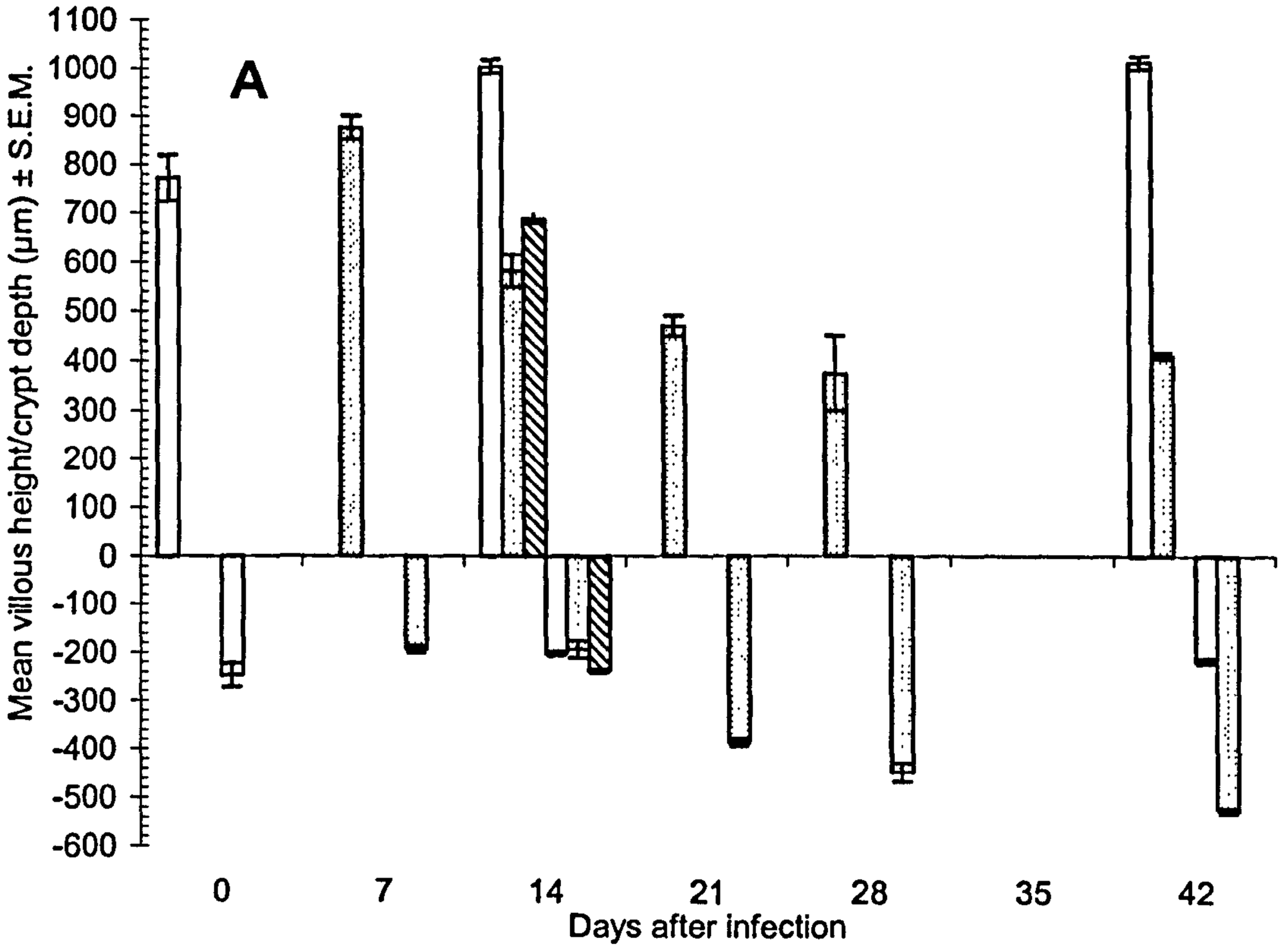
Main effect of time on villi ($F_{5,27}$=55.225, P<0.001)
Main effect of treatment on villi ($F_{1,27}$=317.144, P<0.001)
Interaction between treatment and time ($F_{1,27}$=10.078, P<0.01)
Model R^2=0.958

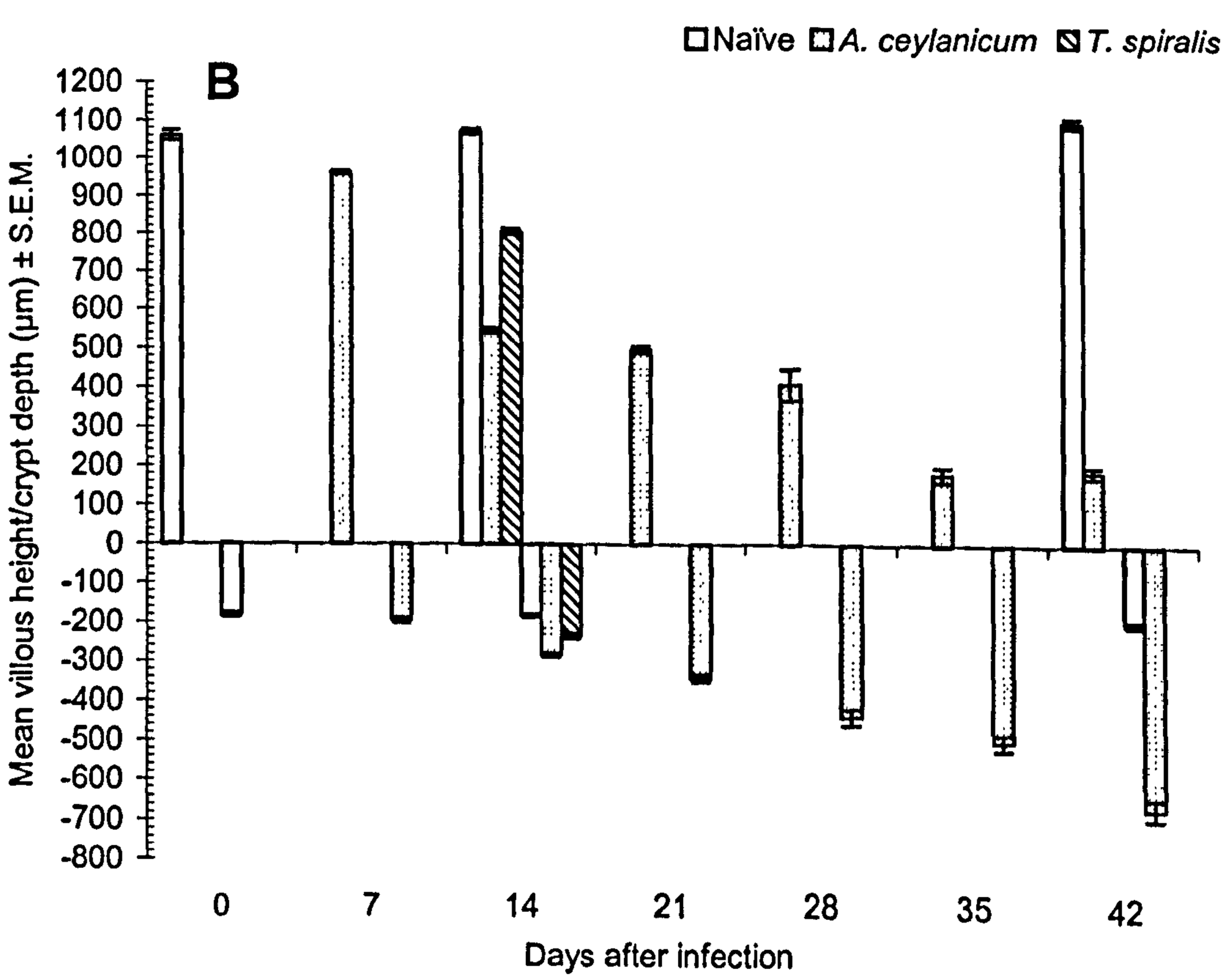
Main effect of time on crypt ($F_{5,27}$=64.475, P<0.001)
Main effect of treatment on crypt ($F_{1,27}$=100.480, P<0.001)
Interaction between treatment and time ($F_{1,27}$=112.493, P<0.001)
Model R^2=0.943

Analysis of data from Experiment 4 with time (7 levels, 0, 7, 14, 21, 28, 35 and 42) and treatment (2 levels, Naïve and A. ceylanicum infection) revealed:

Main effect of time on villi ($F_{6,36}$=212.290, P<0.001)
Main effect of treatment on villi ($F_{1,36}$=1725.778,P<0.001)
Interaction between time and treatment ($F_{1,36}$=121.590, P<0.001)
Model R^2=0.989

Main effect of time on crypts ($F_{6,36}$=89.742, P<0.001)
Main effect of treatment on crypts ($F_{1,36}$=404.671, P<0.001)
Interaction between treatment and time ($F_{1,36}$=167.696, P<0.001)
Model R^2=0.964


Naïve
A. ceylanicum
T. spiralis
A
Mean villous height/crypt depth (µm) ± S.E.M.
1100
1000
900
800
700
600
500
400
300
200
100
0
-100
-200
-300
-400
-500
-600
0
7
14
21
28
35
42
Days after infection

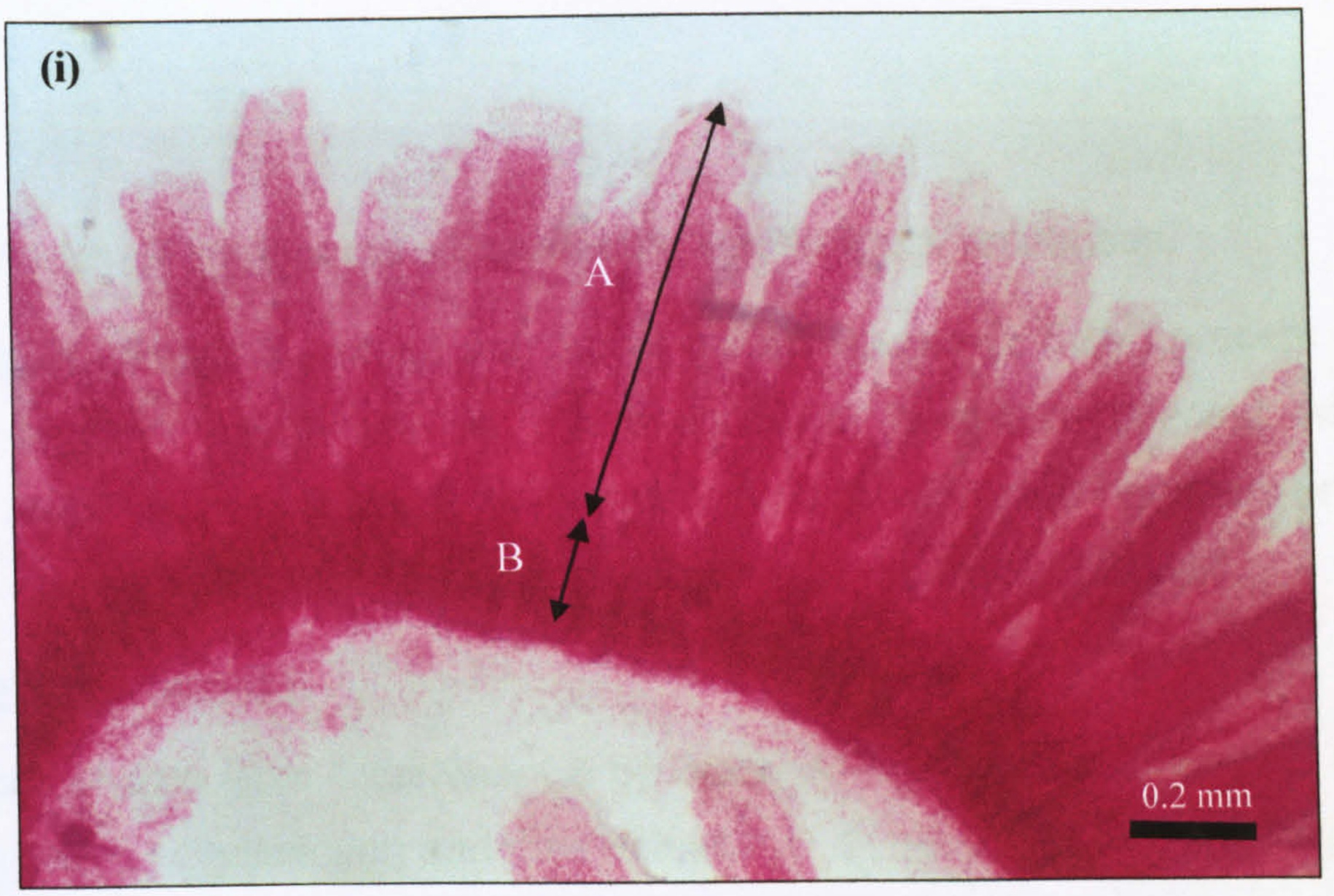

Naïve
A. ceylanicum
T. spiralis
B
Mean villous height/crypt depth (µm) ± S.E.M.
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
-100
-200
-300
-400
-500
-600
-700
-800
0
7
14
21
28
35
42
Days after infection

increased continuously and significantly from 194 µm ± 8.276 on day 7 to 526.250 µm ± 6.884 on day 42 in Experiment 3 and from 195.5 µm ± 7.517 on day 7 to 664 µm ± 26.441 on day 42 post infection in Experiment 4. (r_s=0,918 n=22, P<0.01 and r_s=0,980 n=30, P<0.01, respectively). However, depth was stable and remained short and very similar in naïve uninfected groups in both experiments over the same period (r_s=-0,291 n=13, P=0.336 in Experiment 3 and r_s=0,465 n=15, P=081 in Experiment 4). Moreover, a highly significant interaction between time and treatment on crypt depth was found when 2-way ANOVA were used (Experiment 3. $F_{1,27}$=112.493, P<0.001 and Experiment 4. $F_{1,36}$=167.696, P<0.001).

Sections of hamsters' intestines from Experiment 4, showing the changes in villous height and crypts depth, are illustrated in Figure 3.11.

3.3.2.3 – Cellular division in the crypt of Lieberkuhn

Figure 3.12 (A) (Experiment 3) shows that the number of mitotic figures was higher among infected compared with naïve, control animals. However, there were no significant differences in the mitotic figures counted in the Crypt of Lieberkuhn. (2 way ANOVA, main effect of treatment, $F_{1,24}$=2.373, P=0.137). The higher value of mitotic figures in naïve control hamsters on day 0 again suggests that these animals were suffering from an additional enteric infection at the time the experiment was begun. No interaction was tested in this experiment because of some missing value from day 35 and 42 after infection. On the other hand, cell division in Experiment 4 showed that there were clear differences in the number of mitotic figures between infected and control hamsters (2-way ANOVA, main effect of treatment, $F_{1,36}$=119.215, P<0.001) and a significant interaction between treatment and time ($F_{1,36}$=8.846, P<0.01) indicating a divergent response to treatment with time. However, the interaction was not as marked as in changes associated with villous architecture because mitotic figures appeared to peak on day 28 and declined thereafter (Figure 3.12, B). Nevertheless, there was a significant increase with time across the data-set (r_s=0.753, n= 35, P<0.001) in infected animals, although not in controls



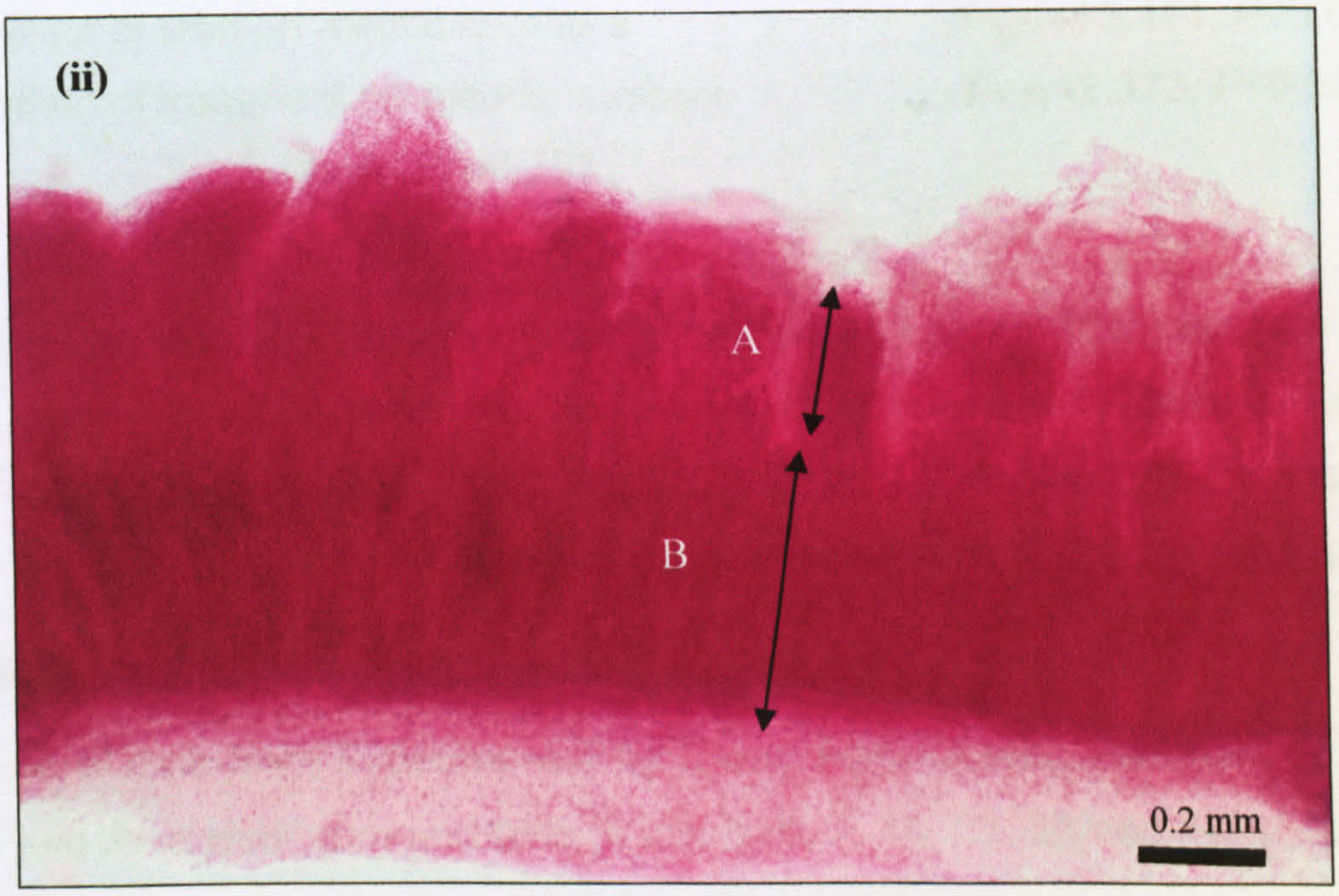


Figure 3.11 – Experiment 4. (Time-course). Changes in the architecture of the small intestine of hamsters infected with *A. ceylanicum*.

(i)- Control uninfected hamsters show long villi (A) and short crypt (B).

(ii)- Infected hamsters with *A. ceylanicum* on day 35 pi show short villi (A) and long crypt (B). (Magnification 100X).

Figure 3.12 - (Time-course experiments). Mitotic figure responses.

(A) Experiment 3 - Mean number of mitotic figures (± SEM) present in the crypt of Lieberkuhn in the intestine of hamsters infected with *A. ceylanicum* on days 7, 14, 21 and 28, and uninfected hamsters on days 0, 14 and 42, and hamsters infected with *T. spiralis* on day 14.

Statistical analysis:

Analysis of data from Experiment 3 by 2-way ANOVA with treatment (2 levels, Naïve and *A. ceylanicum*) and time (6 levels, 0, 7, 14, 21, 28 and 42) revealed

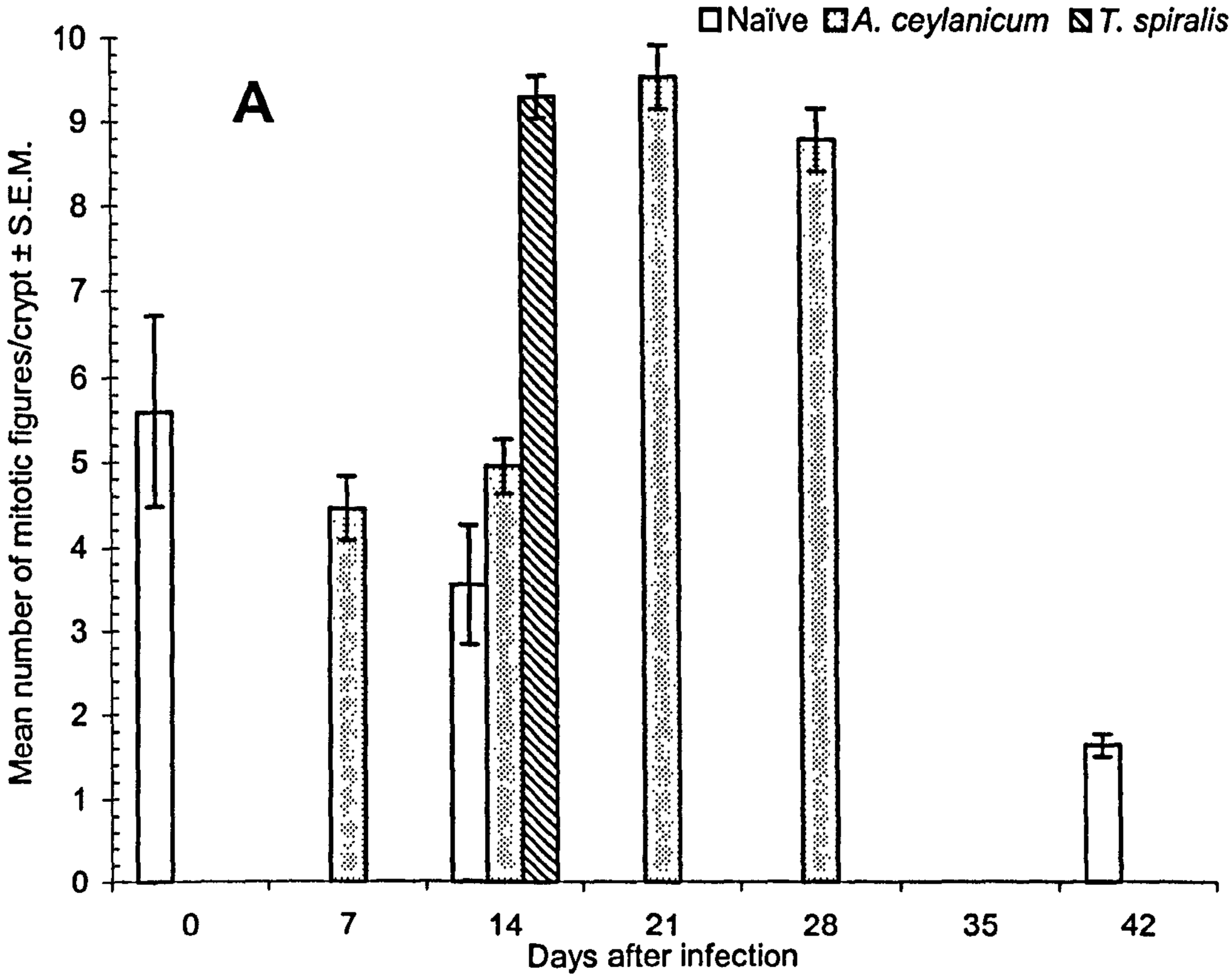
Main effect of time on mitotic numbers ($F_{5,24}$=15.471, P<0.001)
Main effect of treatment on mitotic numbers ($F_{1,24}$=2.373, P<0.001)
Model R^2=0.797

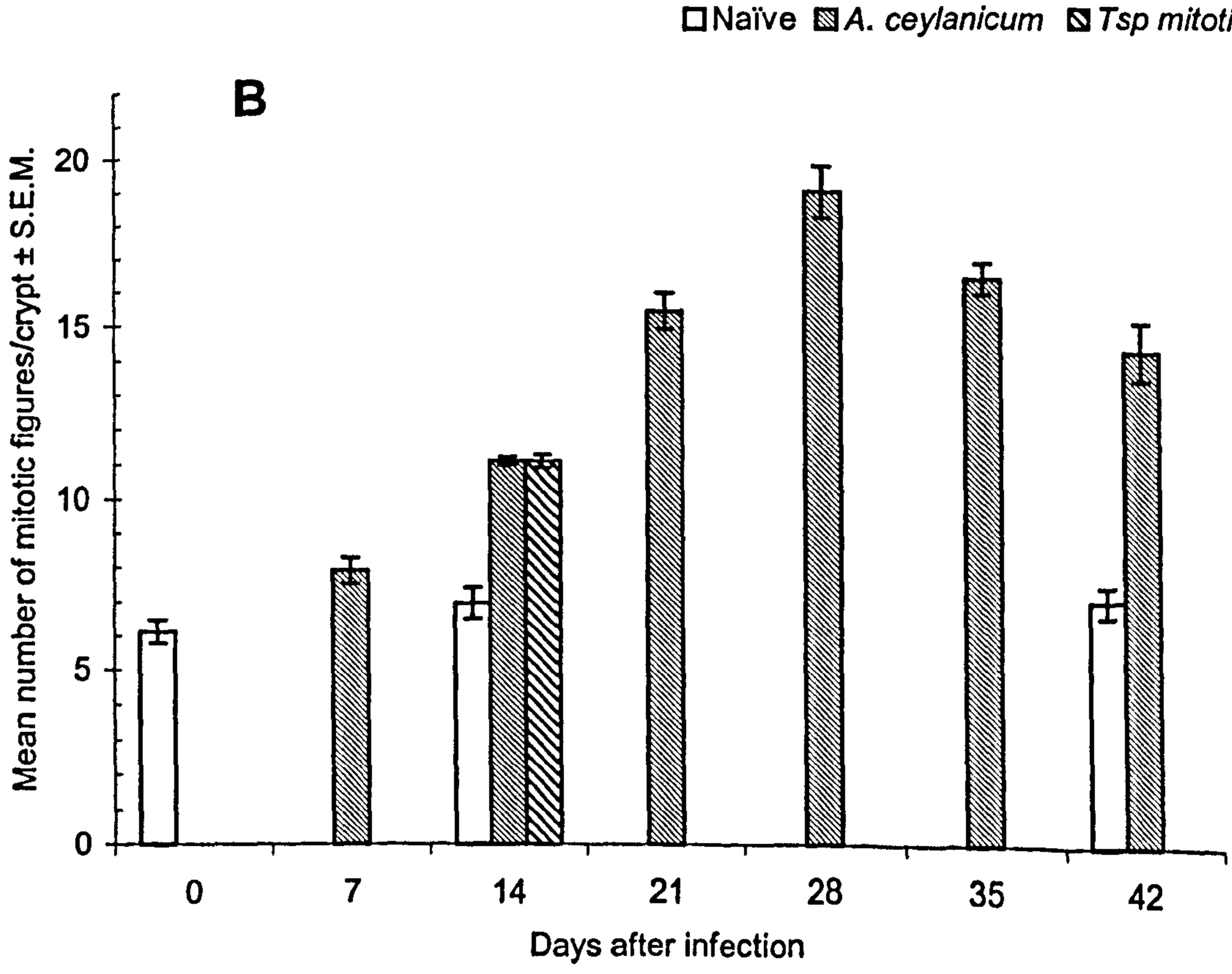
(B) Experiment 4 - Mean number of mitotic figures (± SEM) present in the crypt of Lieberkuhn in the intestine of hamsters infected with *A. ceylanicum* on days 7, 14, 21, 28, 35 and 42, and uninfected hamsters on days 0, 14 and 42, and hamsters infected with *T. spiralis* on day 14.

Statistical analysis:

Analysis of data from Experiment 4 by 2-way ANOVA with treatment (2 levels, naïve and *A. ceylanicum*) and time (6 levels, 0, 7, 14, 21, 28 and 42) showed:

Main effect of time on mitotic ($F_{6,36}$=47.473, P<0.001)
Main effect of treatment on mitotic numbers ($F_{1,36}$=119.215, P<0.001)
Interaction between treatment and time numbers ($F_{1,36}$=8.846, P<0.01)
Model R^2= 0.939


Naïve
A. ceylanicum
T. spiralis
A
Mean number of mitotic figures/crypt ± S.E.M.
0
1
2
3
4
5
6
7
8
9
10
0
7
14
21
28
35
42
Days after infection


Naïve
A. ceylanicum
Tsp mitotic
B
Mean number of mitotic figures/crypt ± S.E.M.
0
5
10
15
20
0
7
14
21
28
35
42
Days after infection

(r_s=0.435, n= 15, P=0.103). In sections of the intestine in Experiment 4, there were higher numbers of mitotic figures in infected animals compared to naïve uninfected groups (Figures 3.13).

3.3.2.4 – Mast cell responses

Mast cell numbers increased with time from a mean of 4.388 cells/VCU on day 7 post infection to a mean of 10.530 cells/VCU in *A. ceylanicum* infected hamsters on day 22 followed by a reduction by day 28 and then by an increase towards the end of Experiment 3 (Figure 3.14 - A). Clear differences were also noticed between infected and control animals (2-way ANOVA, main effect of treatment and interaction between treatment and time were highly significant, $F_{1,27}$= 92.914, P<0.001 and $F_{1,27}$= 17.598, P<0.001, respectively). Similarly, mast cell numbers illustrated in Figure 3.14 (B) (Experiment 4 with illustrated photos of intestinal sections in Figure 3.15) were much higher among infected compared with control animals (2-way ANOVA main effect of treatment, $F_{1,34}$= 37.332, P<0.001). Mast cell numbers increased with time (r_s= 0.457, n=30, P<0.05) in infected animals but not in control (r_s=0.359, n=15, P=0.189), and in the 2-way ANOVA, there was a significant interaction between time and treatment ($F_{1,34}$= 15.902, P<0.001). A similar dip in mast cell numbers, but occurring later compared with Experiment 3, was detected on day 35 before rising to reach the mean of 40.045 cells/mm^{-2} of intestinal tissue.

3.3.2.5 – Goblet cell responses

Goblet cell changes were much like those of mast cells in Experiment 4 (Figure 3.16 - B) but not in Experiment 3 (Figure 3.16 - A). Counts were much higher in infected compared with control animals as seen in Figure 3.17. (the 2-way ANOVA gave main effect of treatment, $F_{1,36}$= 236.541, P<0.001 in Experiment 4 and $F_{1,27}$= 39.490, P<0.001 in Experiment 3). There was also a highly significant interaction between time and treatment in Experiment 4 ($F_{1,36}$= 64.800, P <0.001). The number of goblet cells rose from 87.9 cells/mm^2 ± 6.421

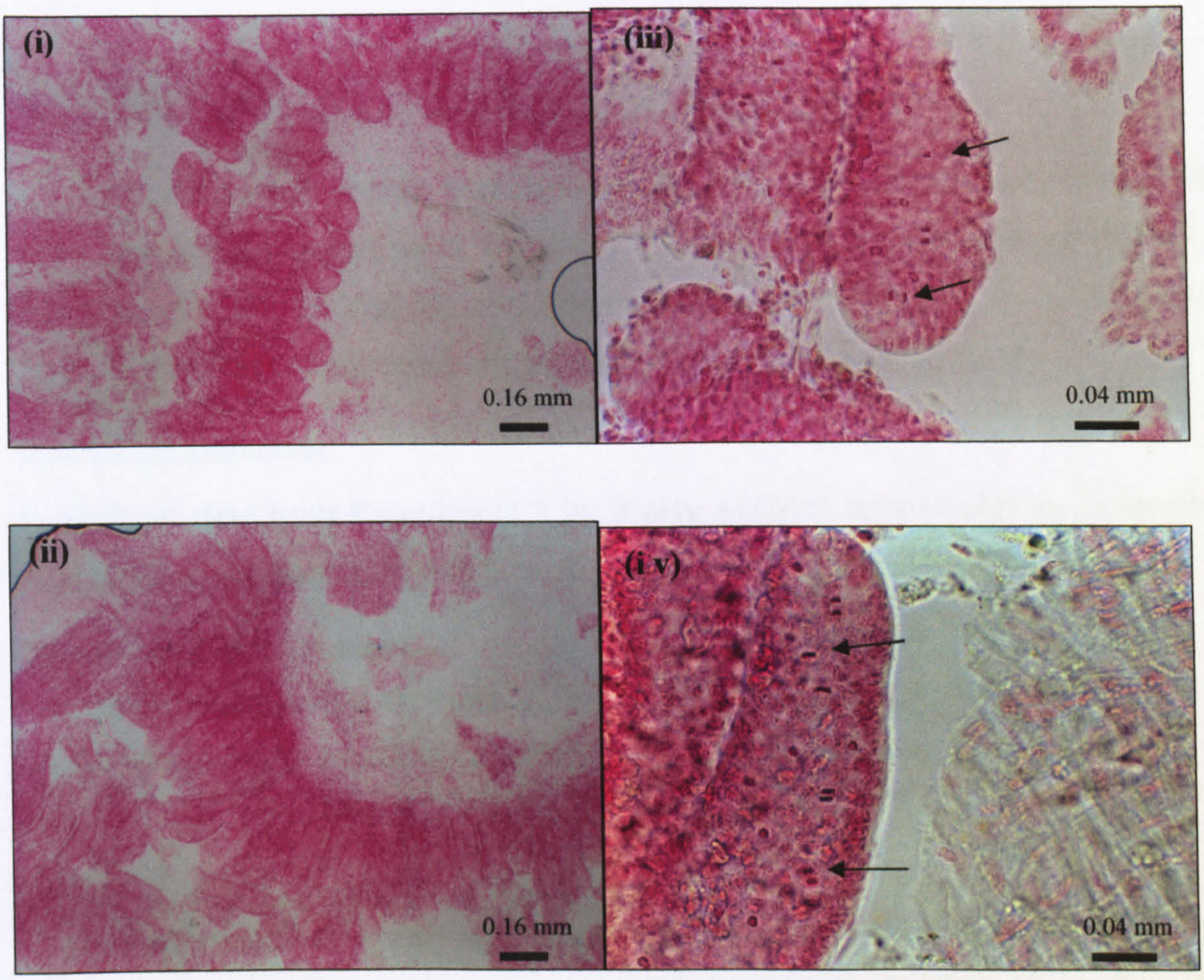


Figure 3.13 – Experiment 4. (Time-course Experiments). Squashed crypts taken from hamsters intestine showing the length of the crypts in

(i)- In Naïve-uninfected hamsters (Magnification 100 X).

(ii)- Hamsters infected with *A. ceylanicum* infection on day 35 (Magnification 100 X).

The number of mitotic figures (arrowed) in the crypt of :

(iv)- Naïve-uninfected hamsters on day 42. (Magnification 400 X)

(iii)- Infected hamsters with A. ceylanicum on day 35. (Magnification 400 X).

Figure 3.14 – (Time-course experiments). Mast cell responses.

(A) Experiment 3 - Mean numbers of Mast cells ± S.E.M. present in VCU of the intestinal tissues of hamsters infected of *A. ceylanicum* on days 7, 14, 21, 28, 35 and 42, and uninfected hamsters (Naïve) on days 0, 14, and 42, and from hamsters infected with *T. spiralis* on day 14.

Statistical analysis:

Analysis of data from Experiment 3 by 2-way ANOVA with treatment (2 levels, Naïve and *A. ceylanicum*) and time (6 levels, 0, 7, 14, 21, 28 and 42) revealed

Main effect if time on mast	($F_{6,27}$=10.916, P<0.001)
Main effect of treatment on mast	($F_{1,27}$=92.914, P<0.001)
Interaction between treatment and time	($F_{1,27}$=17.598, P<0.001)

Model R^2=0.860

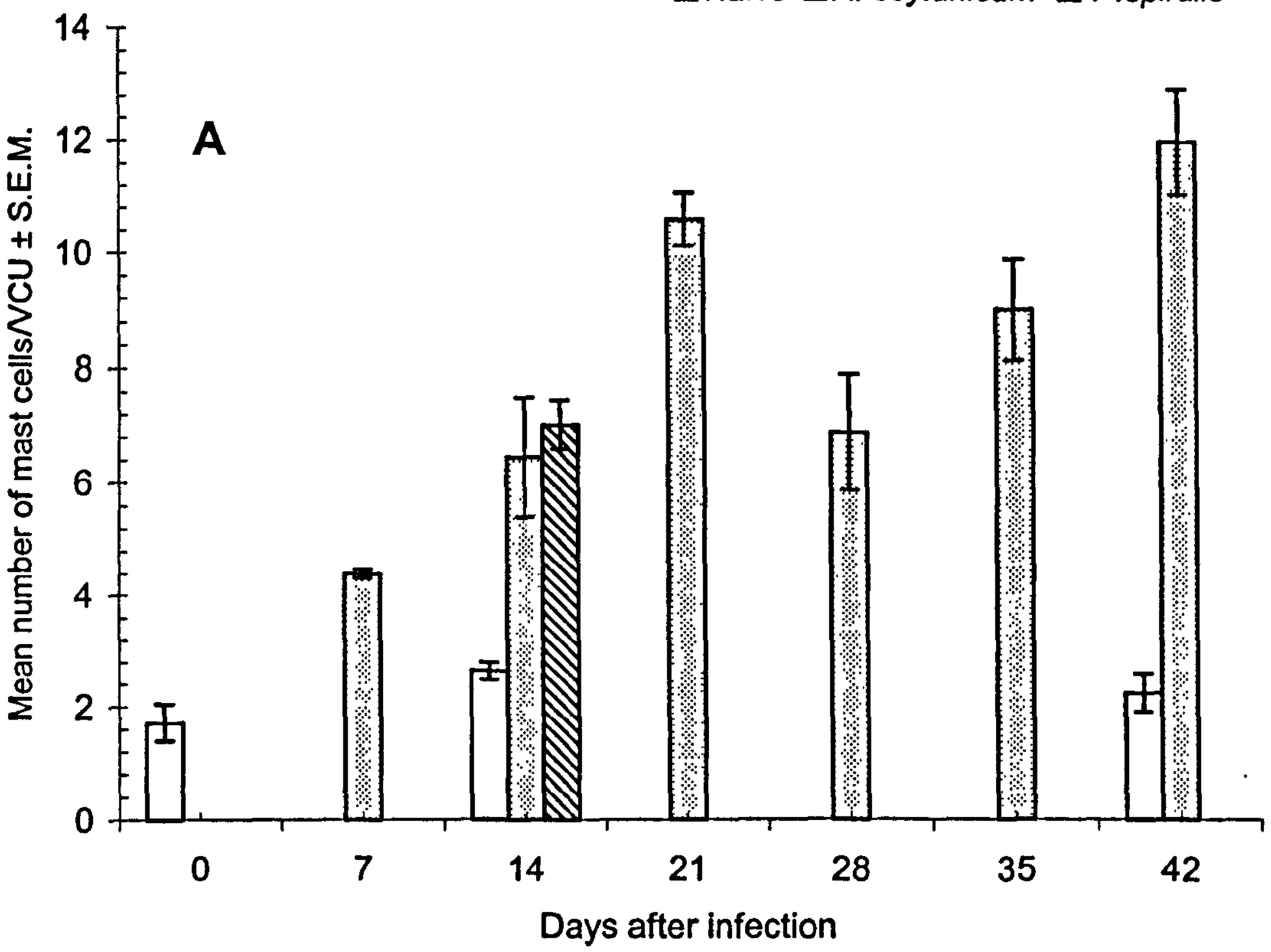
(B) Experiment 4 - Mean numbers of Mast cells ± S.E.M. present in mm² of the intestinal tissues of hamsters infected of *A. ceylanicum* on days 7, 14, 21, 28, 35 and 42, and uninfected hamsters (Naïve) on days 0, 14, and 42, and from hamsters infected with *T. spiralis* on day 14.

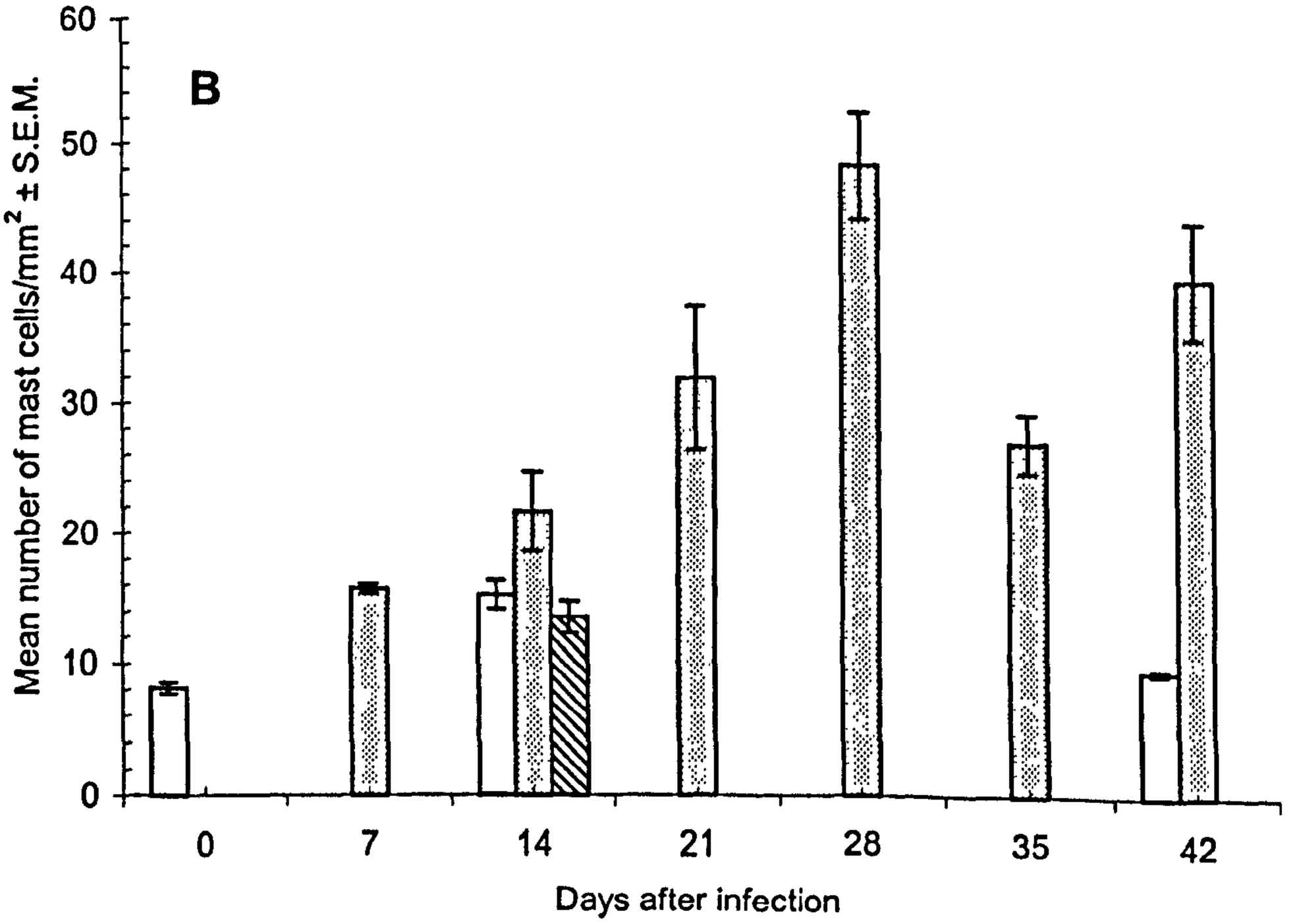
Statistical analysis:

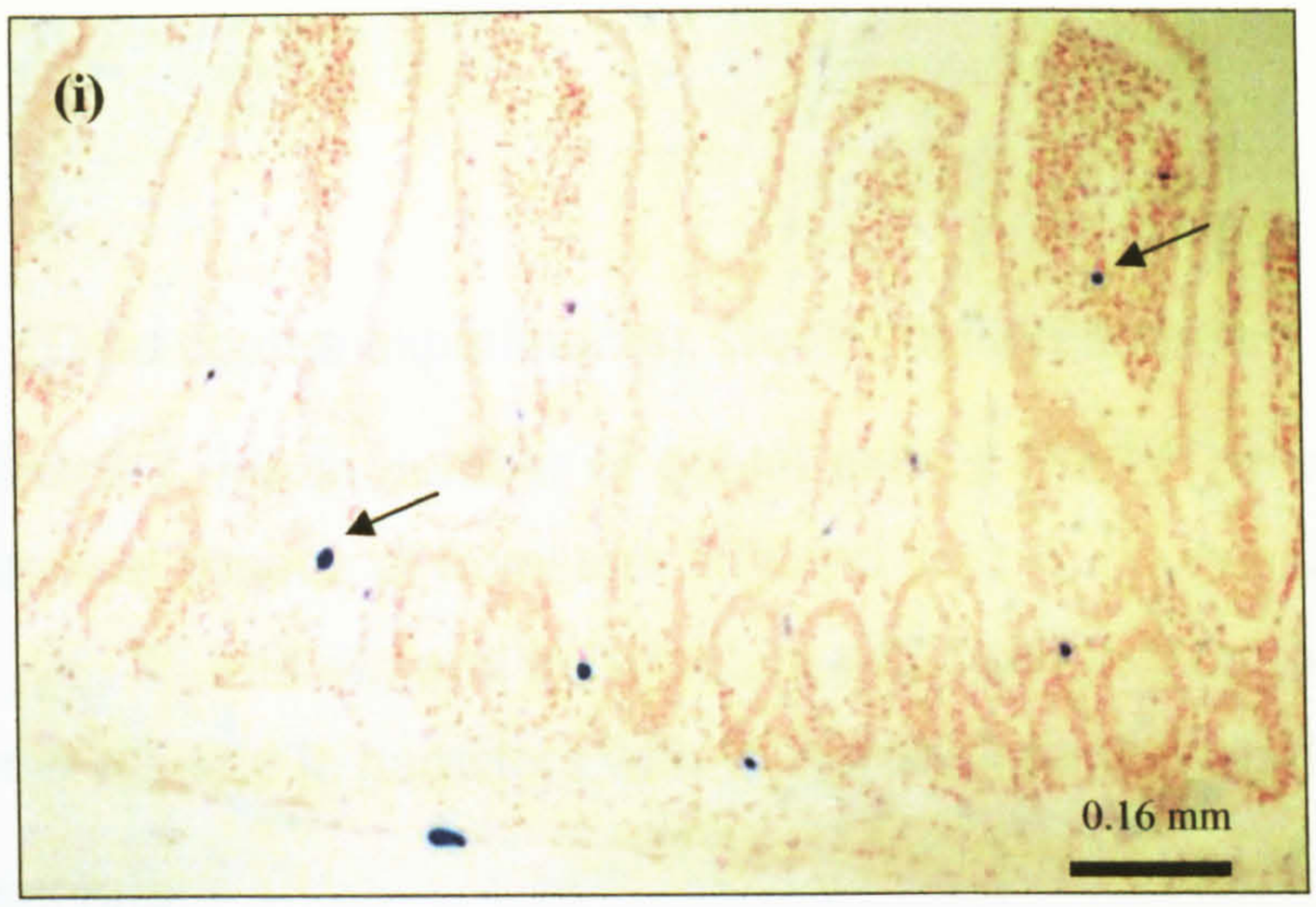
Analysis of data from Experiment 4 by 2-way ANOVA with treatment (2 levels, naïve and *A. ceylanicum*) and time (6 levels, 0, 7, 14, 21, 28 and 42) showed:

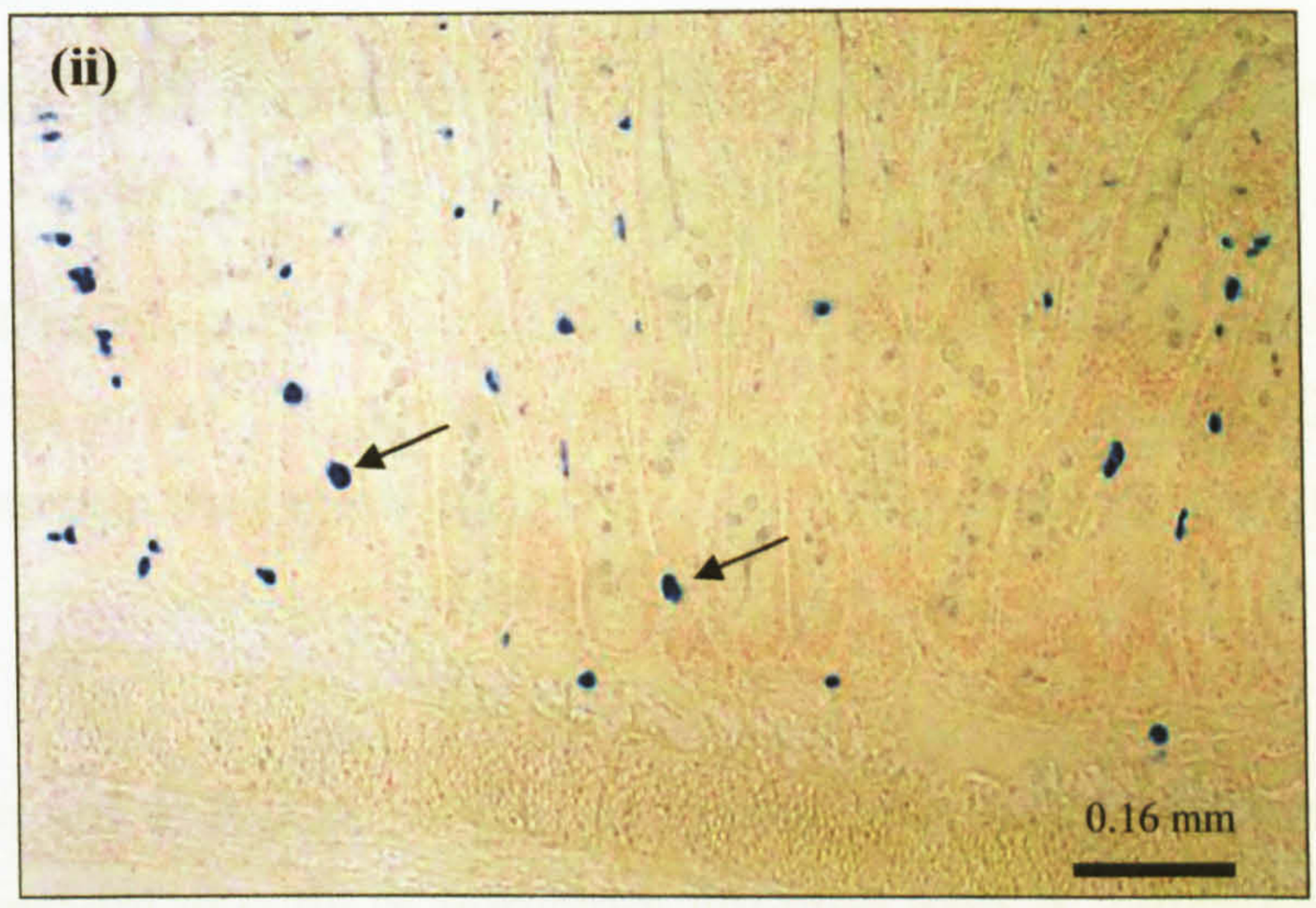
Main effect of time on mast	($F_{6,34}$=9.817, P<0.001)
Main effect of treatment on mast	($F_{1,34}$=37.332, P<0.001)
Interaction between treatment and time	($F_{1,34}$=15.902, P<0.001)

Model R^2= 0.775


Naïve
A. ceylanicum
T .spiralis
A
Mean number of mast cells/VCU ± S.E.M.
0
2
4
6
8
10
12
14
0
7
14
21
28
35
42
Days after infection


B
Mean number of mast cells/mm² ± S.E.M.
0
10
20
30
40
50
60
0
7
14
21
28
35
42
Days after infection





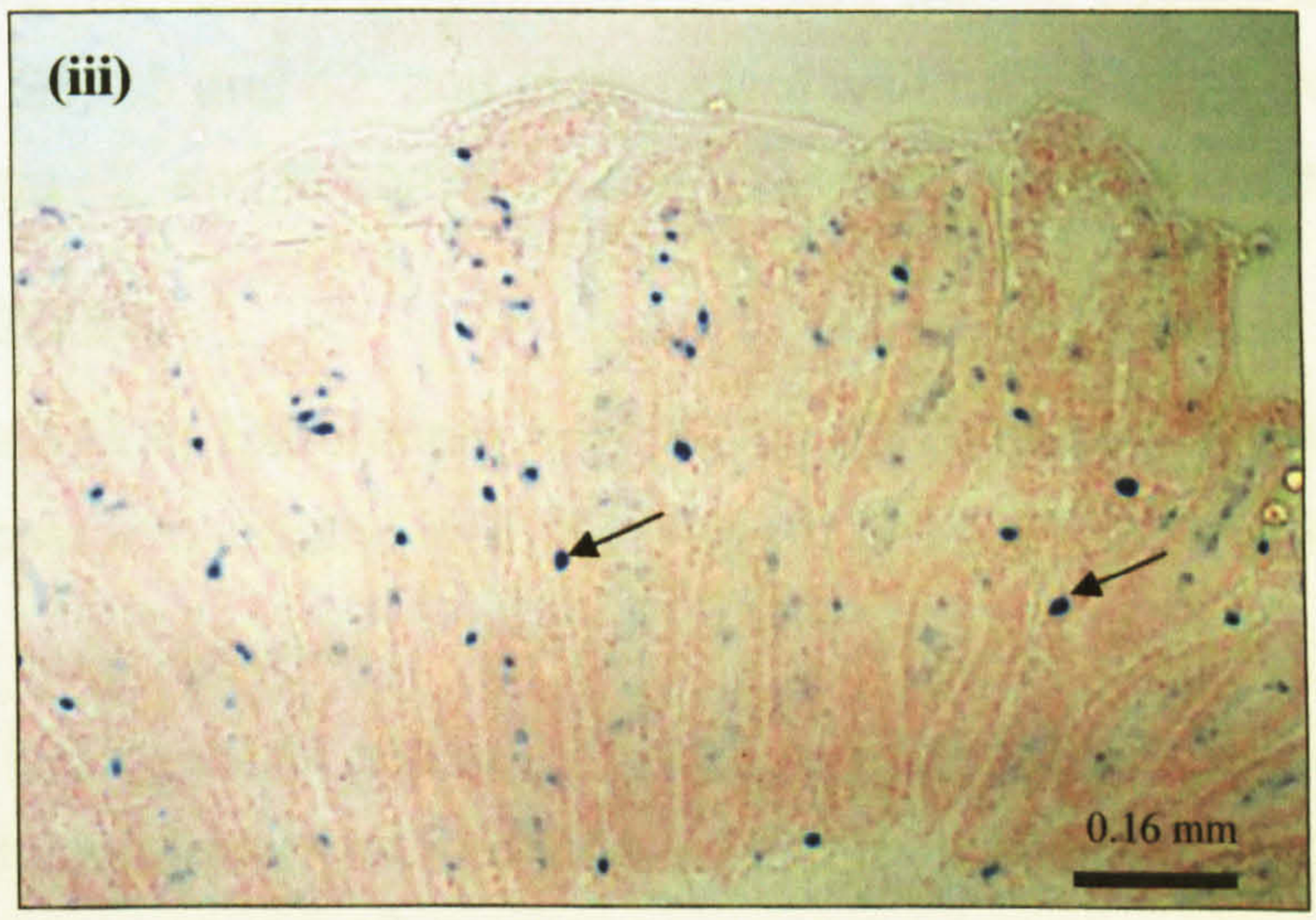


Figure 3.15 – Experiment 4 (Time-course experiments). Mucosal mast cell responses (arrowed) generated during infection with *A. ceylanicum*.

(i)- Intestine of naïve uninfected hamsters on day 14

(ii)- Intestine of hamsters infected with *A. ceylanicum* on day 14

(iii)- Intestine of hamsters infected with *A. ceylanicum* on day 28

(Magnification 100X)

Figure 3.16 – (Time-course experiments). Goblet cells responses.

(A) Experiment 3 -Mean number of goblet cells ± S.E.M. present in VCU in the intestinal tissues of hamsters infected with *A. ceylanicum* on days 7, 14, 21, 28, 35 and 42, and in hamsters with no infection (Naïve) on days 0, 14 and 42, and in hamsters with *T. spiralis* infection on day14.

Statistical analysis:

Analysis of data from Experiment 3 by 2-way ANOVA with treatment (2 levels, Naïve and *A. ceylanicum*) and time (6 levels, 0, 7, 14, 21, 28 and 42) revealed

Main effect if time on goblet	($F_{6,27}$=19.300, P<0.001)
Main effect of treatment on goblet	($F_{1,27}$=39.490, P<0.001)
Interaction between treatment and time	($F_{1,27}$=5.376, P<0.001)

Model R^2=0.875

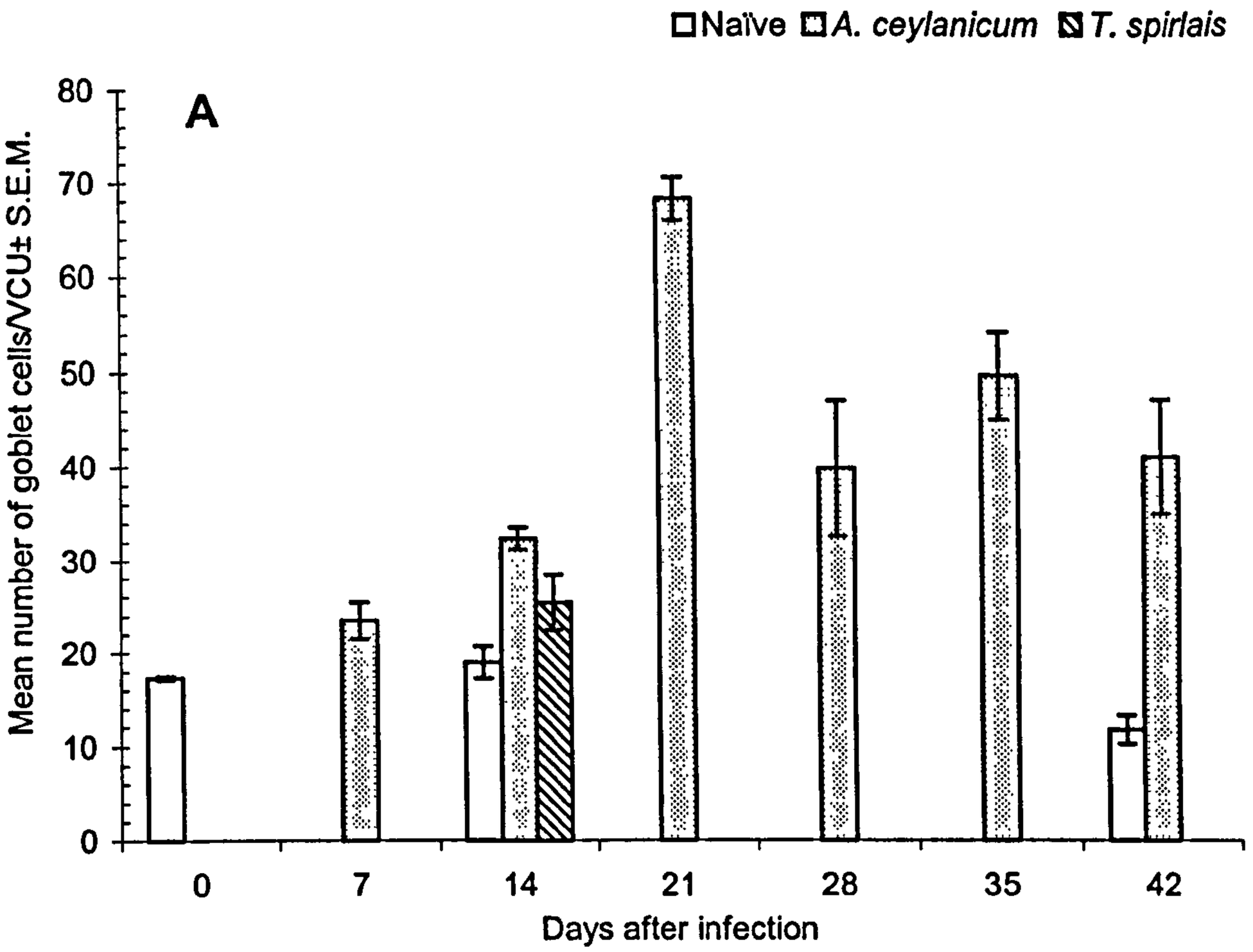
(B) Experiment 4 -Mean number of goblet cells ± S.E.M. present in mm² in the intestinal tissues of hamsters infected with *A. ceylanicum* on days 7, 14, 21, 28, 35 and 42, and in hamsters with no infection (Naïve) on days 0, 14 and 42, and in hamsters with *T. spiralis* infection on day14.

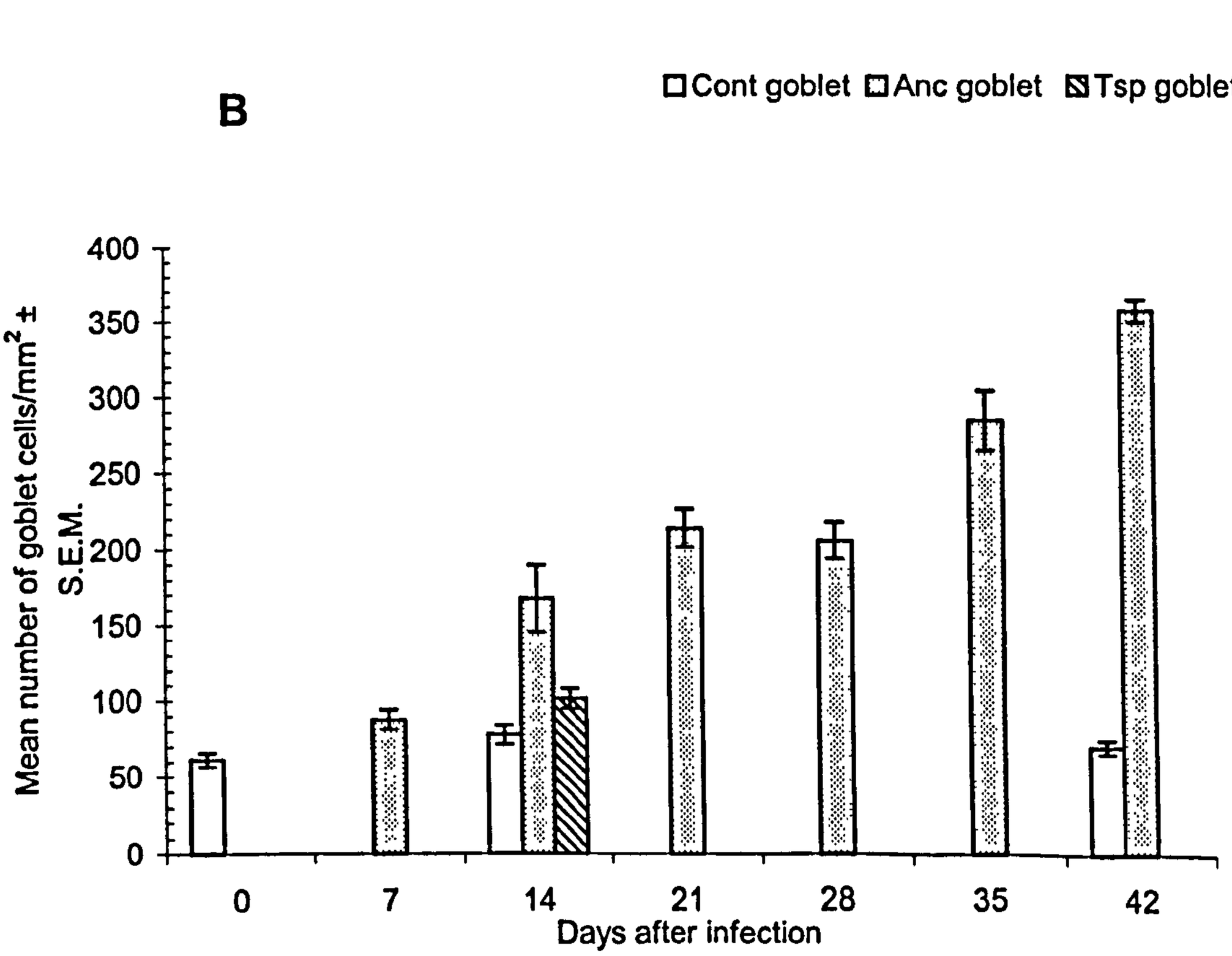
Statistical analysis:

Analysis of data from Experiment 4 by 2-way ANOVA with treatment (2 levels, naïve and *A. ceylanicum*) and time (6 levels, 0, 7, 14, 21, 28 and 42) showed:

Main effect of time on goblet	($F_{6,36}$=37.515, P<0.001)
Main effect of treatment on goblet	($F_{1,36}$=236.541, P<0.001)
Interaction between treatment and time	($F_{1,36}$=64.800, P<0.001)

Model R^2= 0.930


A
Naïve
A. ceylanicum
T. spirlais
Mean number of goblet cells/VCU± S.E.M.
80
70
60
50
40
30
20
10
0
0
7
14
21
28
35
42
Days after infection


B
Cont goblet
Anc goblet
Tsp goblet
Mean number of goblet cells/mm² ± S.E.M.
400
350
300
250
200
150
100
50
0
0
7
14
21
28
35
42
Days after infection

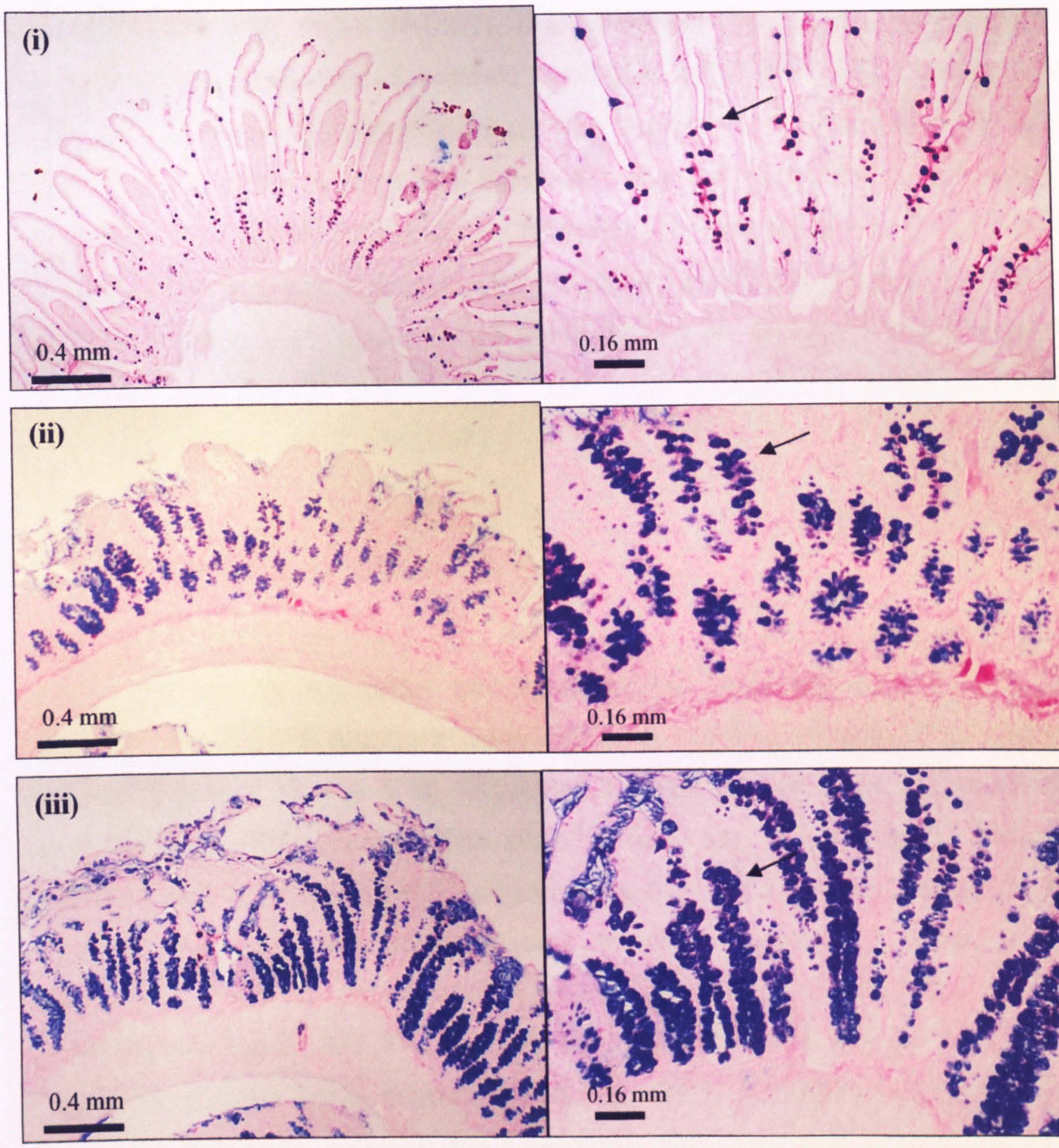


Figure 3.17 – Experiment 4 (Time-course experiments). Goblet cell responses generated during infection with *A. ceylanicum*.

(i)- Intestine of naïve-uninfected hamsters on day 14.

(ii)- Intestine of hamsters infected with A. ceylanicum on day 28.

(iii)- Intestine of hamsters infected with A. ceylanicum on day 35.

(Magnifications are 40 X on the left and 100 X on the right).

on day 7 and continued with a dip on day 28 to reach 357.258 cells/mm^2 of tissue ± 7.4 on day 42. Whilst this increase was highly significant in infected hamsters (r_s=0.894, n=30, P<0.01), there was no significant time-dependant increase in the naïve control animals (r_s=0.359, n=15, P=0.189). Similarly the interaction between treatment and time was significant in experiment 3 (Figure 3.16 – A), this analysis showed a significant effect of treatment ($F_{1,27}$= 39.490, P<0.001) with a significant interaction between time and treatment ($F_{1,27}$= 5.376, P<0.05) on the number of goblet cells. However, goblet cell changes in infected groups over time were less marked (r_s=0.460, n=24, P<0.05). There were no significant changes in goblet cells with time among the naïve-uninfected animals (r_s=-0.563, n=12, P=0.057).

3.3.2.6 – Paneth cell responses

In contrast to the other cell types during Experiments 3 and 4, the observed Paneth cell numbers were higher in controls compared to infected animals (Figure 3.18 – A & B). However, the numbers of cells fluctuated with large variation within groups. The effect of time and the interaction between treatment and time on Paneth cell numbers from analysis by 2-way ANOVA indicated that there was no significant effects ($F_{6,29}$=1.528, P=0.204 and $F_{1,29}$=0.053, P=0.820 respectively). However, treatment affected Paneth cells significantly ($F_{1,29}$=21.321, P<0.001). In Experiment 4 there was an increases in Paneth cells in the naïve animals (r_s= 0.568, n=15, P=0.027) and there was a significant difference between the treatments and time (2-way ANOVA, main effect of treatment, $F_{1,36}$= 33.544, P<0.001 and main effect of time, $F_{6,36}$=5.616, P<0.001) although the interaction between treatment and time was not significant (2-way ANOVA, $F_{1,36}$= 2.521, P=0.121). Despite the overall trends, both groups showed lower average values on day 42 compared with day 14. It is interesting to note that in Experiment 3, where worm burdens were heavier, but significant worm loss occurred, Paneth cell numbers fell by >50% by day 7 and then steadily increased with time. In contrast, in Experiment 4 in which fewer worms established initially and no loss of worms was observed, Paneth cell numbers fell with time (Figure 1.19).

Figure 3.18 – (Time-course experiments). Paneth cell responses.

(A) Experiment 3 -Mean number of Paneth cells ± S.E.M. in the crypt of Lieberkuhn of hamsters infected with *A. ceylanicum* on days 7, 14, 21, 28, 35 and 42, and in naïve uninfected hamsters on days 0, 14 and 42, and in hamsters with *T. spiralis* infection on day 14.

Statistical analysis:

Analysis of data from Experiment 3 by 2-way ANOVA with treatment (2 levels, Naïve and *A. ceylanicum*) and time (6 levels, 0, 7, 14, 21, 28, 35 and 42) revealed:

Main effect if time on Paneth	($F_{6,29}$=1.528, P=0.204)
Main effect of treatment on Paneth	($F_{1,29}$=21.321, P<0.001)
Interaction between treatment and time	($F_{1,29}$=0.053, P=0.820)

Model R^2=0.538

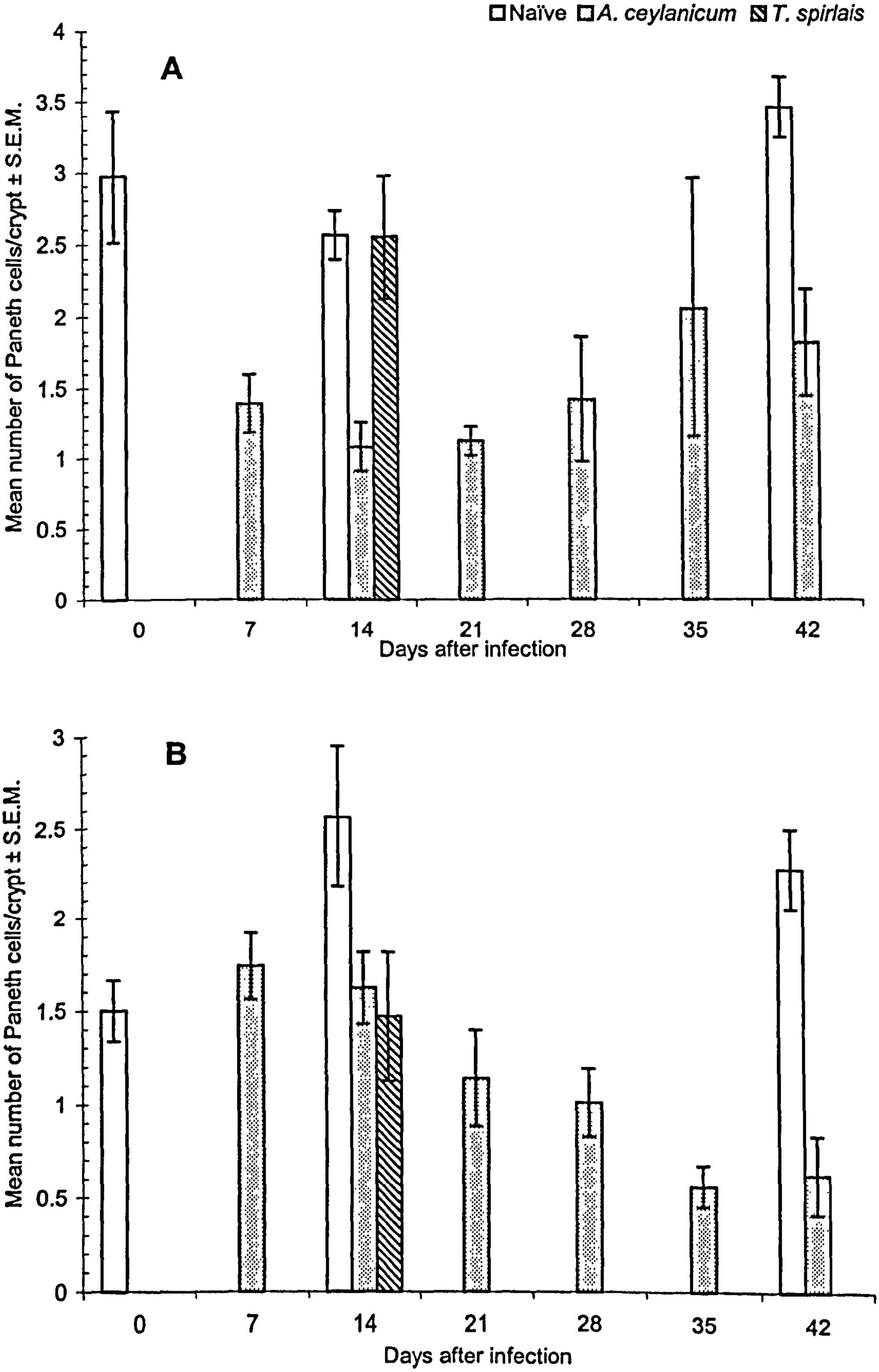
(B) Experiment 3 -Mean number of Paneth cells ± S.E.M. in the crypt of Lieberkuhn of hamsters infected with *A. ceylanicum* on days 7, 14, 21, 28, 35 and 42, and in naïve uninfected hamsters on days 0, 14 and 42, and in hamsters with *T. spiralis* infection on day 14

Statistical analysis:

Analysis of data from Experiment 4 by 2-way ANOVA with treatment (2 levels, naïve and *A. ceylanicum*) and time (6 levels, 0, 7, 14, 21, 28, 35 and 42) revealed:

Main effect of time on Paneth	($F_{6,36}$=5.616, P<0.001)
Main effect of treatment on Paneth	($F_{1,36}$=33.544, P<0.001)
Interaction between treatment and time	($F_{1,36}$=2.521, P=0.121)

Model R^2= 0.607


Naïve
A. ceylanicum
T. spirlais
A
Mean number of Paneth cells/crypt ± S.E.M.
4
3.5
3
2.5
2
1.5
1
0.5
0
0
7
14
21
28
35
42
Days after infection
B
Mean number of Paneth cells/crypt ± S.E.M.
3
2.5
2
1.5
1
0.5
0
0
7
14
21
28
35
42
Days after infection

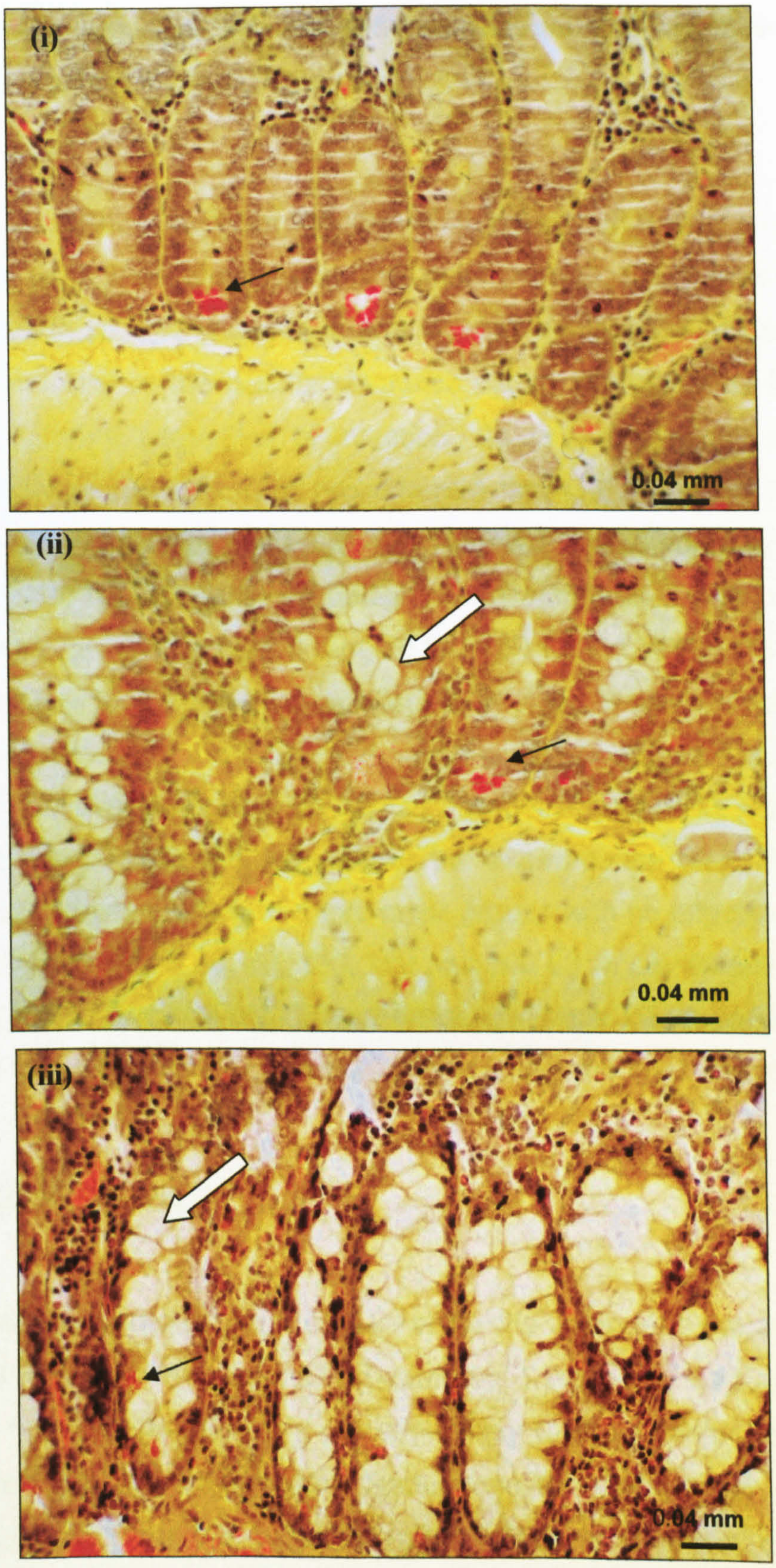


Figure 3.19 – Section of intestinal tissue show the Paneth cells (pointed with black arrows) in the naïve uninfected hamsters on day 14 pi **(i)** and in hamsters infected with *A. ceylanicum* on 14 pi **(ii)** and on day 35 pi **(iii)**. The white arrows indicate the position of goblet cells. (Magnifications are 200 X).

3.3.3 – The relationships between the mucosal cellular responses and the intensity of worm burdens (Dose-response).

It was of interest to determine the relationship between the extent of the cellular and structural changes in the mucosa and the intensity of worm burdens. Therefore, Experiments 5 and 6 were designed and carried out to determine changes in the cellular response within the hamsters intestines in relation to different intensities of infection. As in the previous section, two experiments were carried out at two different times. Experiment 5 comprised fifty female DSN hamsters aged between 10 and 12 weeks old, arranged in 10 different groups. The experimental design is shown in table 3.5. As can be seen hamsters were given doses of L3 ranging from 50 to 250, and were either killed on day 12 or day 30. Only the lighter infection (100 L3) was allowed to survive to day 30, and therefore the range of doses examined on day 12 is greater than that on day 30. Experiment 6 was carried out similarly at a different time from Experiment 5 and Table 3.6.summarises the experimental plan. Thirty female age-matched hamsters were arranged in six groups with no infection for the first group and then doses of 30, 60, 100, 150 and 200 L3 *A. ceylanicum* for each of the other groups respectively. These hamsters were killed on day 20 after infection for worm counts and histological analysis of the intestinal mucosa.

3.3.3.1 – Worm recovery

The data presented in Figures 3.20 show the worm burdens at recovery. It was found that the worm burdens in Experiment 5 were much as expected with increasing mean worm recovery corresponding to increasing dose of larvae administered. However, there were fluctuations in the numbers of the adult worms recovered from the hamsters after infection. A and C illustrate the number of larvae given and the mean number of adults worms recovered at autopsy on days 12 and 30 respectively from experiment 5 while B summarises the data from Experiment 6. A low number of worms were recovered in Experiment 6. Because these experiments were deigned to assess the

Table 3.5 – Experiment 5 (Dose-response experiments). The effect of varying worm burdens on the mucosal immune responses to infection with *A. ceylanicum* in hamsters.

No. of hamsters	Dose of L3 given	Mean no. of worms recovered	Day killed
5	Nil	0	12
5	Nil	0	30
5	50 L3 *A.ceylanicum*	14.6 ± 2.600	12
5	50 L3 *A. ceylanicum*	14.6 ± 1.778	30
5	100 L3 *A. ceylanicum*	21.6 ± 3.945	12
5	100 L3 *A. ceylanicum*	12.25 ± 4.697	30
5	150 L3 *A. ceylanicum*	34.4 ± 3.586	12*
5	200 L3 *A. ceylanicum*	40.8 ± 11.972	12*
5	250 L3 *A. ceylanicum*	44.6 ± 18.705	12*
5	250 L3 *T. spiralis*	84 ± 6.173	12*

* Infections of the dose above 150 (L3) are difficult to be sustained after day 20 pi as animals will suffer.

Table 3.6 – Experiment 6 (Dose-response experiments). The effect of varying worm burdens on the mucosal immune responses to infection with *A. ceylanicum* in hamsters.

No. of hamsters	Dose of L3 given	Mean no. of worms recovered	Day killed
5	Nil	0	20
5	30 L3 *A. ceylanicum*	10 ± 3.362	20
5	60 L3 *A. ceylanicum*	9 ± 2.746	20
5	100 L3 *A. ceylanicum*	18 ± 3.286	20
5	150 L3 *A. ceylanicum*	16 ± 2.015	20
5	200 L3 *A. ceylanicum*	18 ± 5.161	20

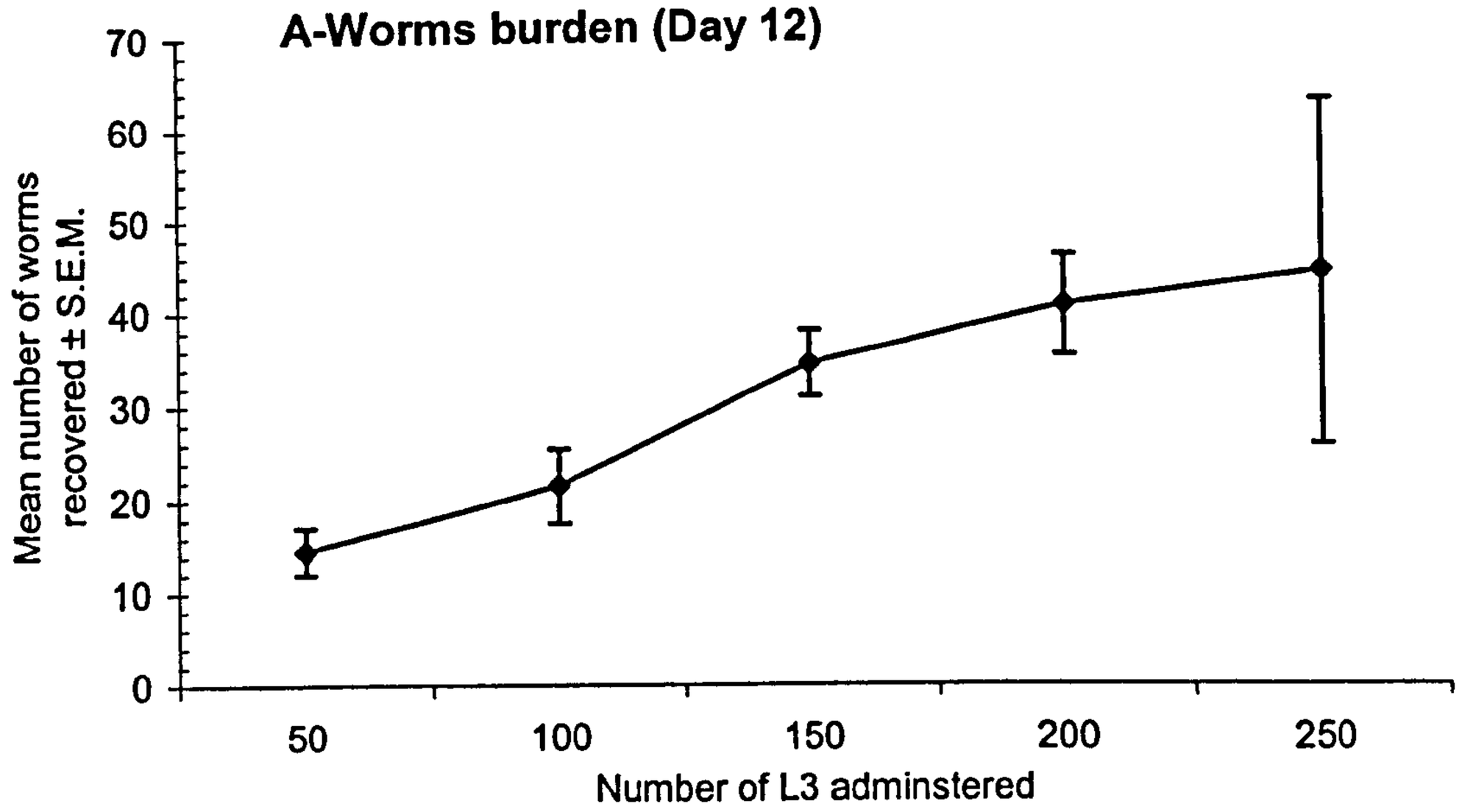
Note: These L3 doses of *A.ceylanicum* were selected to include a variety of doses within the range from low (less than 50 L3) to high (250 L3).

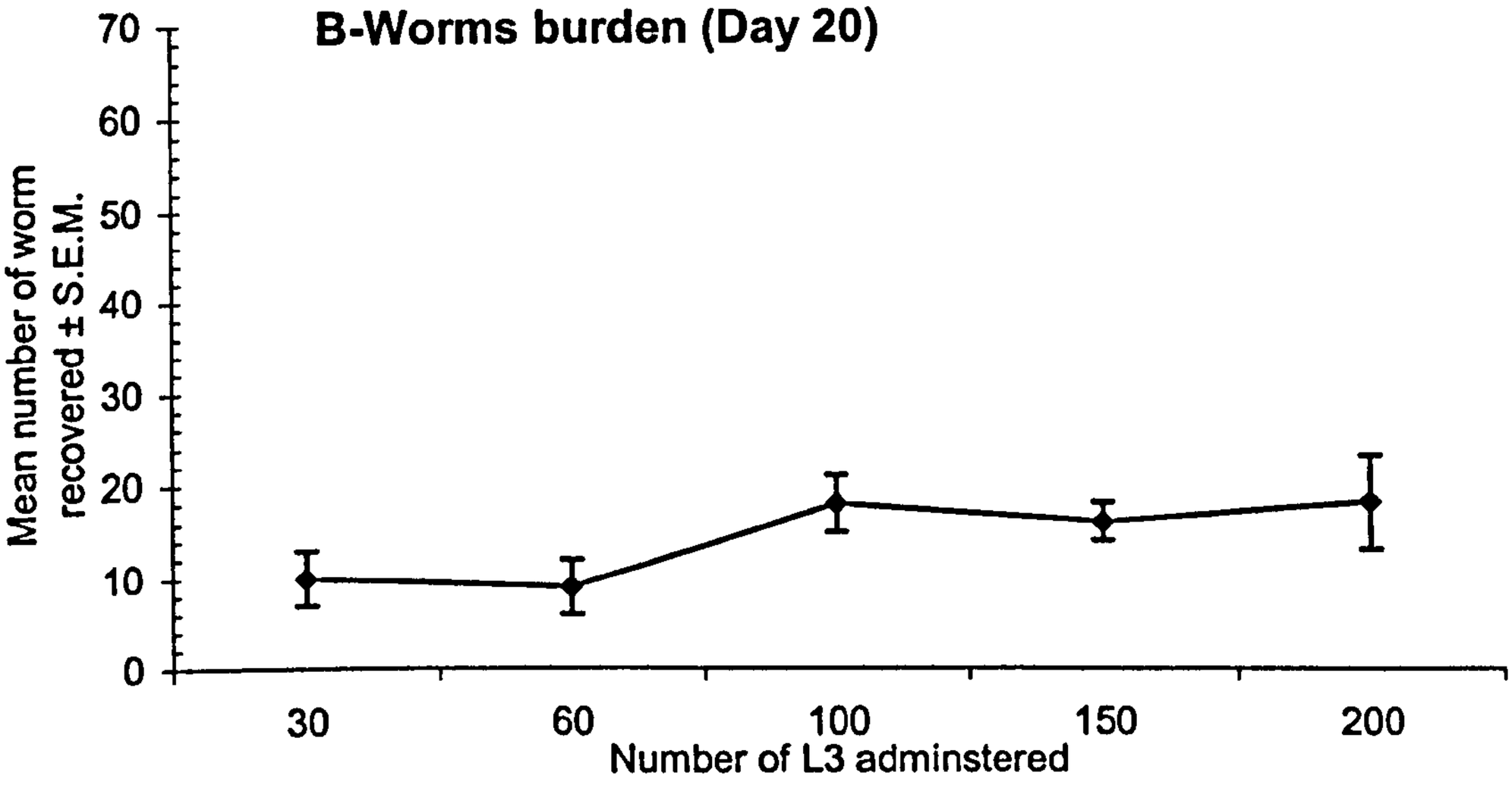
Figure 3.20 – (Dose-response experiments). Mean number of adult *A. ceylanicum* worm recovered from hamsters intestine.

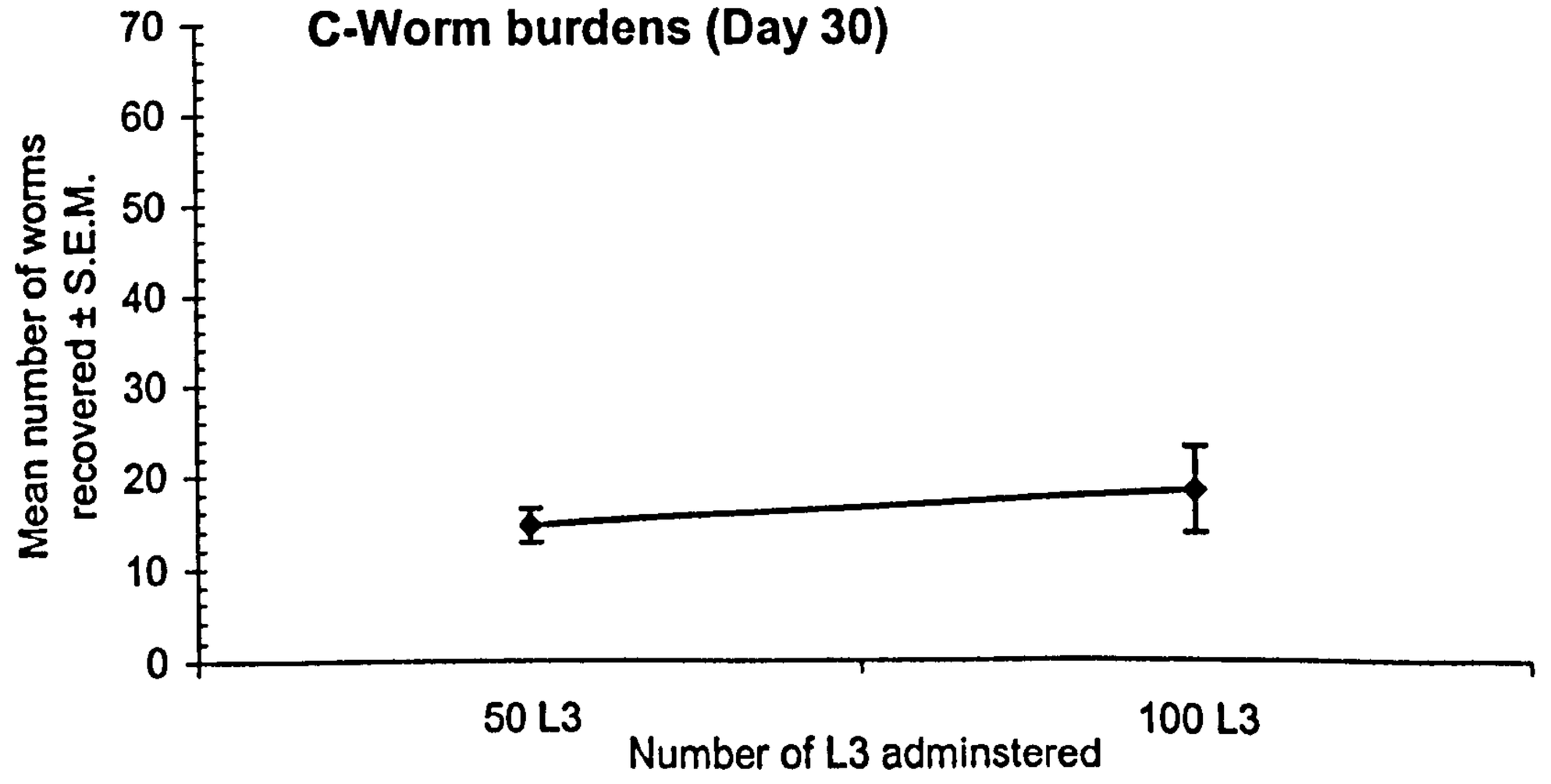
A- Worm burden from hamsters on day 12 (Experiment 5).

B- Worm burden from hamsters on day 20 (Experiment 6).

C- Worm burden from hamsters on day 30 (Experiment 5).


A-Worms burden (Day 12)
Mean number of worms recovered ± S.E.M.
70
60
50
40
30
20
10
0
50
100
150
200
250
Number of L3 adminstered


B-Worms burden (Day 20)
Mean number of worm recovered ± S.E.M.
70
60
50
40
30
20
10
0
30
60
100
150
200
Number of L3 adminstered


C-Worm burdens (Day 30)
Mean number of worms recovered ± S.E.M.
70
60
50
40
30
20
10
0
50 L3
100 L3
Number of L3 adminstered

relationships between the number of worms harboured and other parameters, the analysis that follows is based on correlation between worm recovery and the intensity of cellular responses.

3.3.3.2 – Mucosal architecture and cell division

As in the previous experiments, changes in the architecture of the small intestine during primary infection with *A. ceylanicum* were detected. Analysis was conducted by the Spearman Rank order correlation test (data from Experiment 5 and 6). In addition, in Experiment 5 where animals were subjected to two doses (50 L3 and 100 L3) and controls were culled at 2 different times after infection, 2-way ANOVA was employed to test for an interaction between dose and time.

Villous height

(Figure 3.21 – A) shows that there was no significant correlation between the number of worms recovered and the villous height on day 12 (r_s=0.239, n=30, *P*=0.203) whereas, the relationship between the number of worms recovered and villous height was found to be significant on day 30 as illustrated in Figure 3.21.– C (r_s= -0.688, *n*=15, *P*<0.01). Correlation test, which were also conducted to examine the effect of worm burdens on villous height in Experiment 6 on day 20 (Figure 3.21 – B) also indicated a highly significant effect (r_s=-0.807, *n*=30, *P*<0.001). When 2-way ANOVA was conducted on the results from Experiment 5, (Treatment, 3 levels corresponding to uninfected animals and those given 50L3 and 100L; time, 2 levels corresponding to days 12 and 30) a highly significant interactions between time and treatment on villous height was detected ($F_{2,24}$=73.394, *P*<0.001) (Figure 3.21 - D).

Crypt depth

Similarly, the correlation between the depth of the Crypts and the number of worms recovered was examined on day 12 (Figure 3.22 - A) and found to be not significant (r_s=0.183, *n*=30, *P*=0.334). Changes on day 20 in Experiment 6 and on day 30 in Experiment 5 (Figure 3.22 - B and C, respectively) were found to be highly significant (r_s=0.628, n=15, P<0.05 and r_s=-0.741, *n*=30, *P*=0.001, respectively). Again, 2-way ANOVA were conducted on the results from

Figure 3.21 – (Dose-response experiments). Relationship between the mean number of worms recovered and the villous height following infection of hamsters with different doses of L3 *A. ceylanicum*.

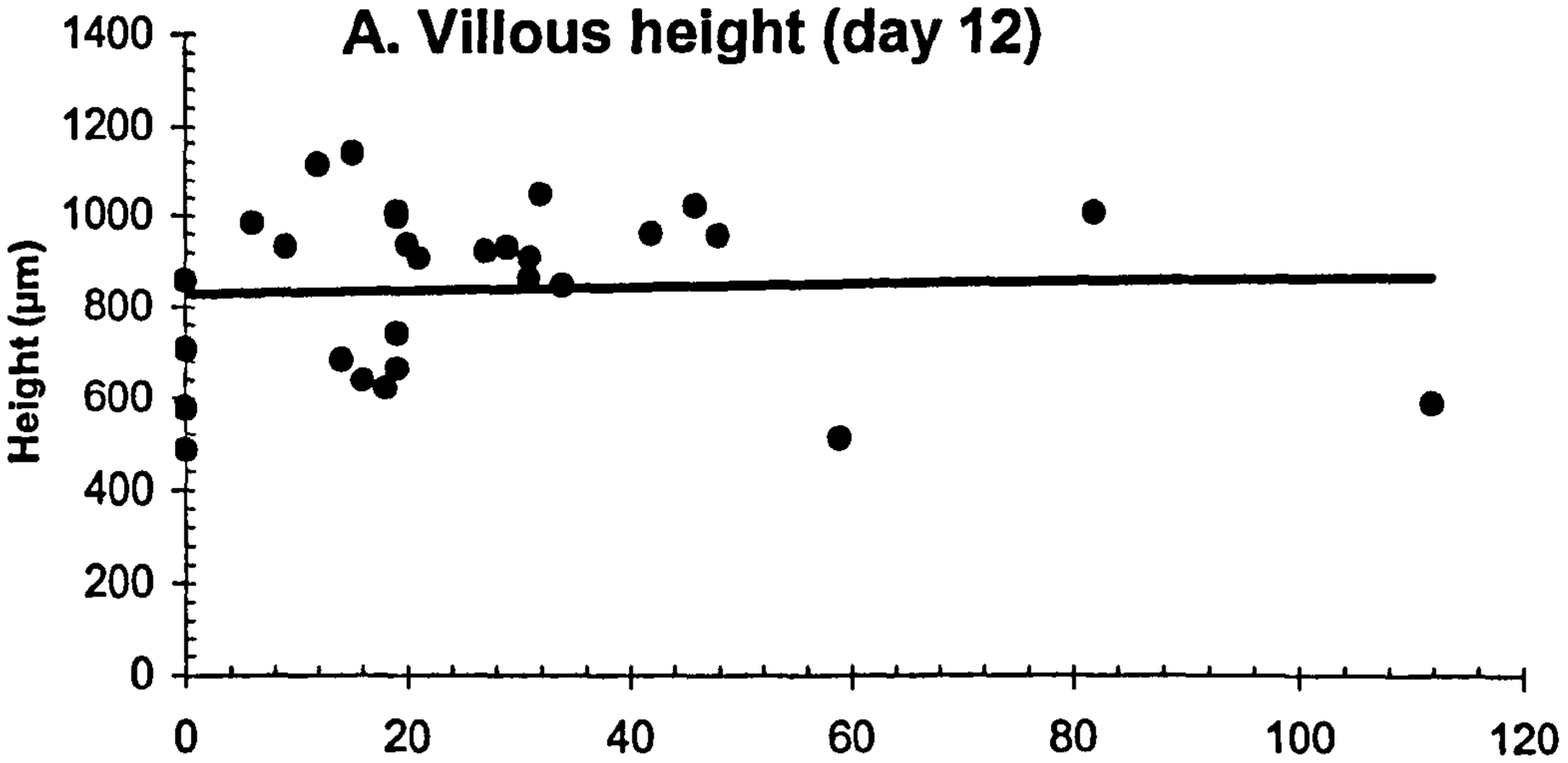
A-Villous height (µm) in experiment 5 (day 12).

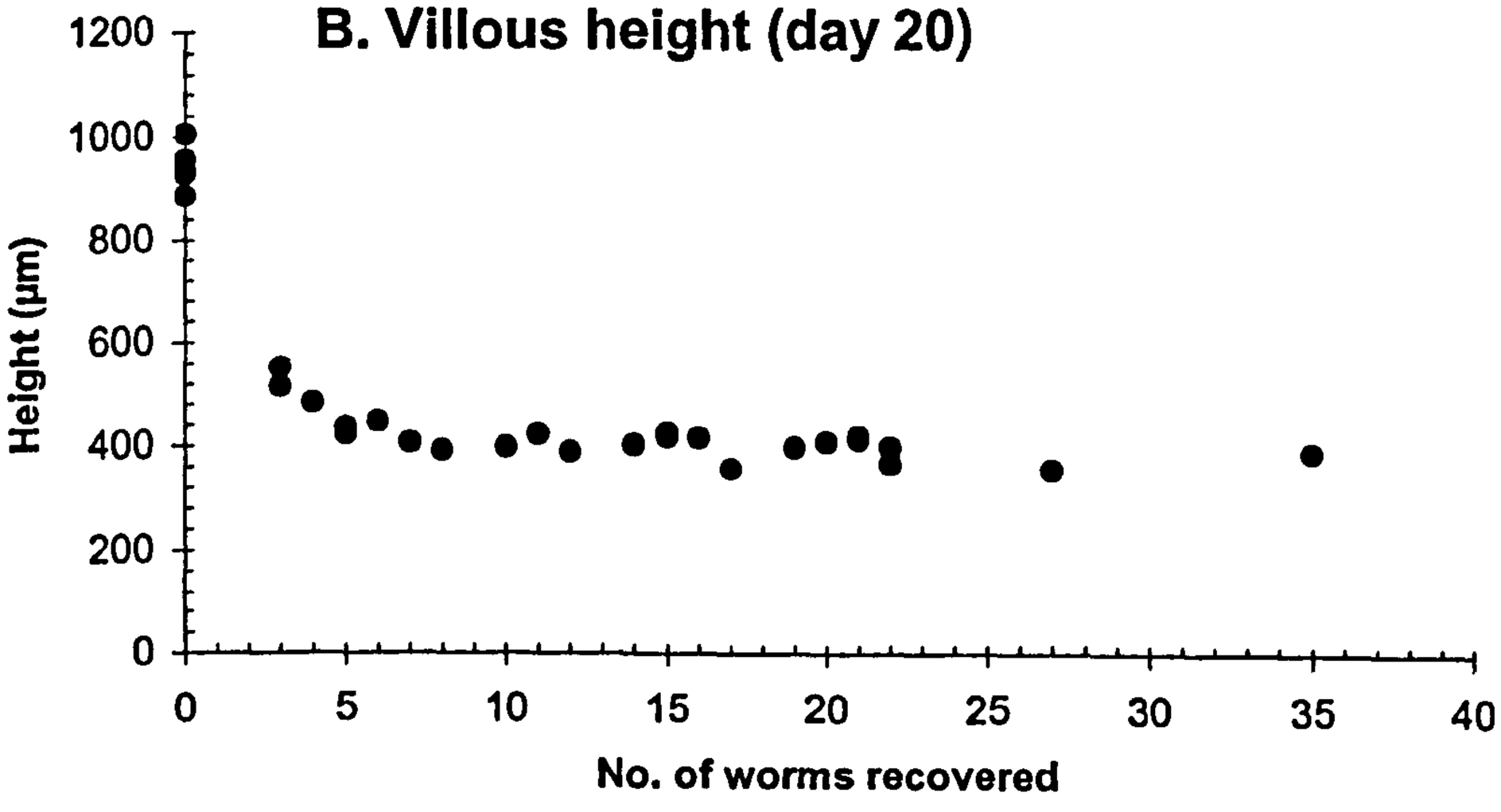
B-Villous height (µm) in experiment 6 (day 20).

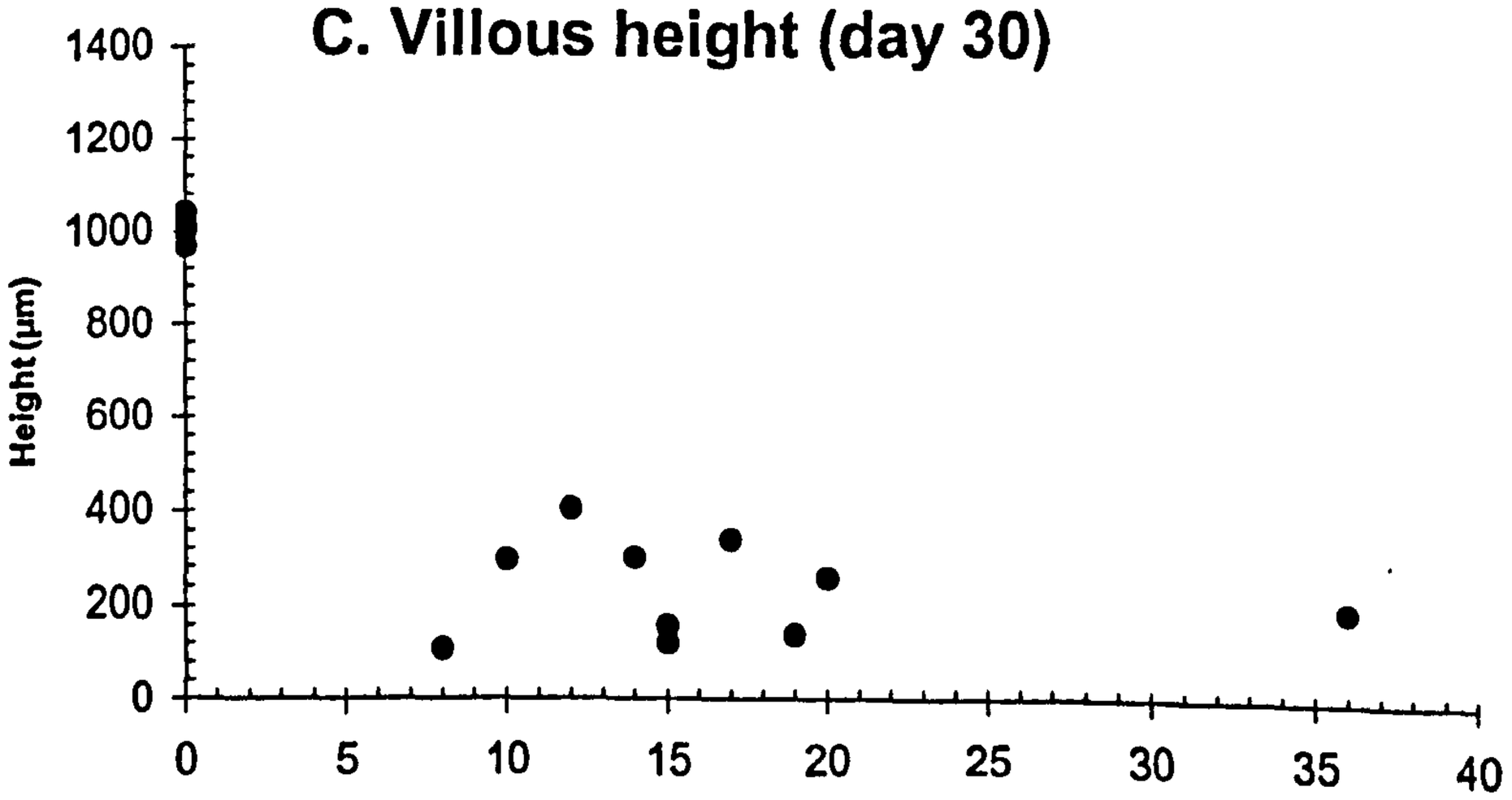
C-Villous height (µm) of experiment 5 (day 30).

Statistical analysis by correlation coefficient test

On day 12 PI (experiment 5)	r_s=0.239, n=30, *P*=0.203
On day 20 PI (experiment 6)	r_s=-0.807, *n*=30, *P*<0.001
On day 30 PI (experiment 5)	r_s= -0.688, *n*=15, *P*=0.0045


A. Villous height (day 12)
Height (µm)
1400
1200
1000
800
600
400
200
0
0
20
40
60
80
100
120


B. Villous height (day 20)
Height (µm)
1200
1000
800
600
400
200
0
0
5
10
15
20
25
30
35
40
No. of worms recovered


C. Villous height (day 30)
Height (µm)
1400
1200
1000
800
600
400
200
0
0
5
10
15
20
25
30
35
40

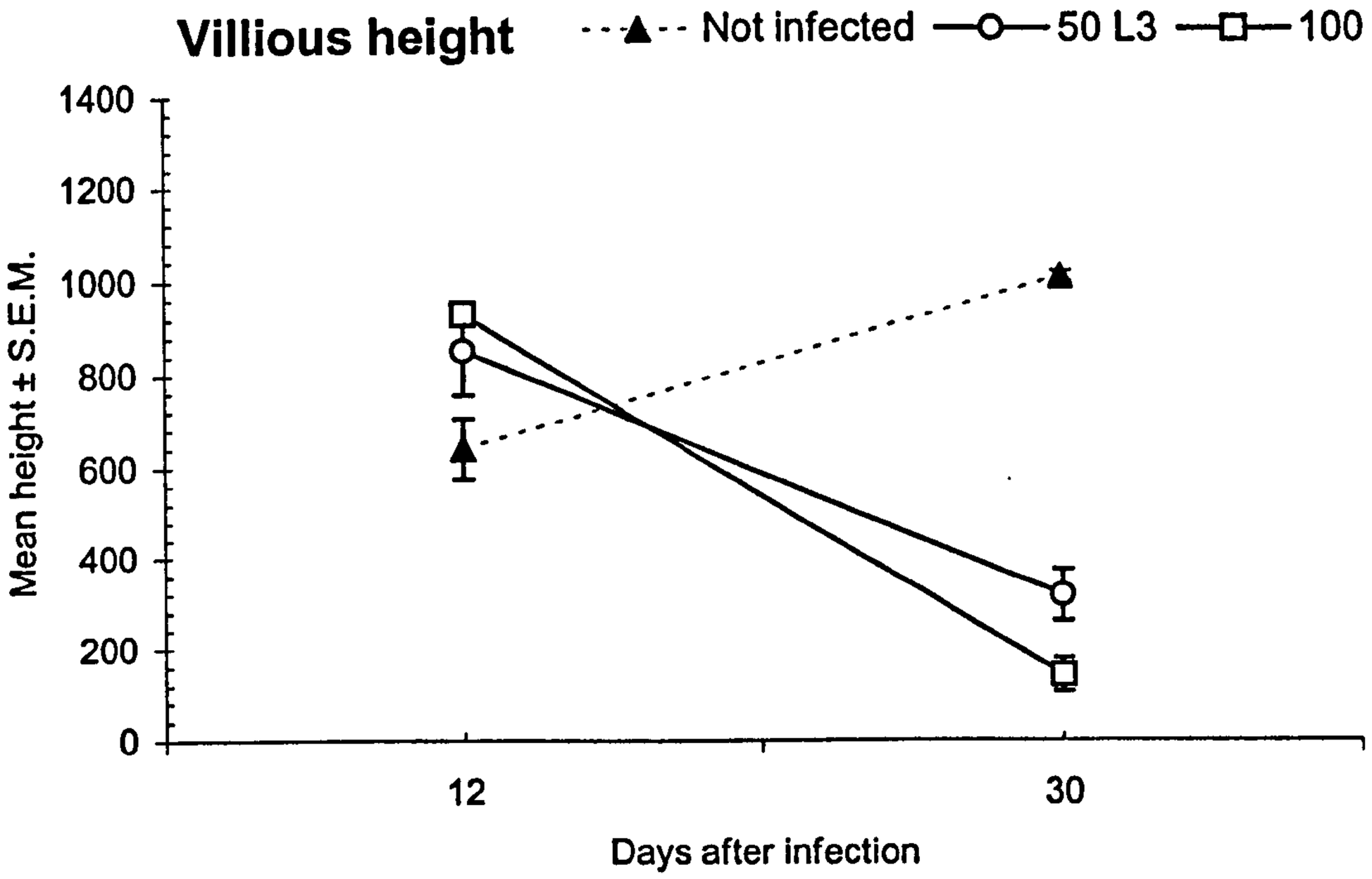


Figure 3.21 - D Experiment 5. (Dose-response experiments). Comparison of villous height during primary infection at different doses of *A. ceylanicum* (Naïve, 50 L3 and 100 L3) and on two different days (12 and 30) after infection.

Statistical analysis

Data analysis of this experiment by 2-way ANOVA with time (2 levels, days 12 and 30 revealed) and treatment (3 levels, Naïve, 50 and 100 L3 *A.ceylanicum*) showed:

Main effect of time ($F_{1,24}$=61.221, P<0.001)

Main effect of treatment ($F_{2,24}$=18.711, P<0.001)

Interaction between treatment and time ($F_{2,24}$=73.394, P<0.001)

Model R^2=0.892

Experiment 5 (Figure 3.22 - D) and this indicated a significant main effect of time and treatment on the Crypts ($F_{1,24}$=840.953, P<0.001 and $F_{2,24}$=290.271, P<0.001, respectively) with a significant interaction between treatment and time ($F_{2,24}$=213.478, P<0.001).

3.3.3.3 – Cellular division in the crypt of Lieberkuhn

From Figure 3.23 - A, it can be seen that the numbers of mitotic figures in the crypts were not significantly affected by the variation in worm burdens recovered on day 12 of Experiment 5 (r_s=-0.049, n=30, P=0.798). However, a highly significance correlation between the number of mitotic figures and worm burdens was detected on day 30 (r_s=0.727, n=15, P<0.01) in the same experiment (Figure 3.23 - C) and on day 20 (r_s=0.620, n=30, P<0.001) of Experiment 6 (Figure 3.23 - B). 2-way ANOVA was also used (day 12 and 30) in Experiment 5 to analyse differences between treatments with time. It was found that there was a highly significant main effect of time ($F_{1,24}$=9.589, P<0.01) and treatment ($F_{2,24}$=67.864, P<0.001). In addition, there was a significant interaction between time and treatment on the cellular response in the Crypts (Figure 3.23 - D) at different treatments (Naïve, 50L3 and 100L3) and on different times (day 12 and 30) ($F_{2,24}$=23.751, P<0.001) after infection.

3.3.3.4 – Mast cell responses

Figure 3.24 – (A) shows that there was no correlation between mast cell numbers and the number of adult worms recovered from hamsters on day 12 (r_s=0.185, n=29, P=0.336) while this relationship approached significance on day 30 (r_s=0.484, n=15, P=0.06) in Experiment 5 (Figure 3.24 - C). The correlation on day 20 in Experiment 5 (Figures 3.24 - B) was highly significant (r_s=0.725, n=30, P<0.001). However, the 2-way ANOVA of the data from Experiment 5 showed that there was a significant overall effect of treatment (50 L3 and 100 L3) and time (day 12 and 30) and a significant interactions between there factors. Increases in mast cells on day 12 from the mean of 11.4 ± 1.9

Figure 3.22 – (Dose-response experiments). Relationship between the number of worms recovered and the depth of the Crypt following infection of hamsters with different doses of L3 *A. ceylanicum*.

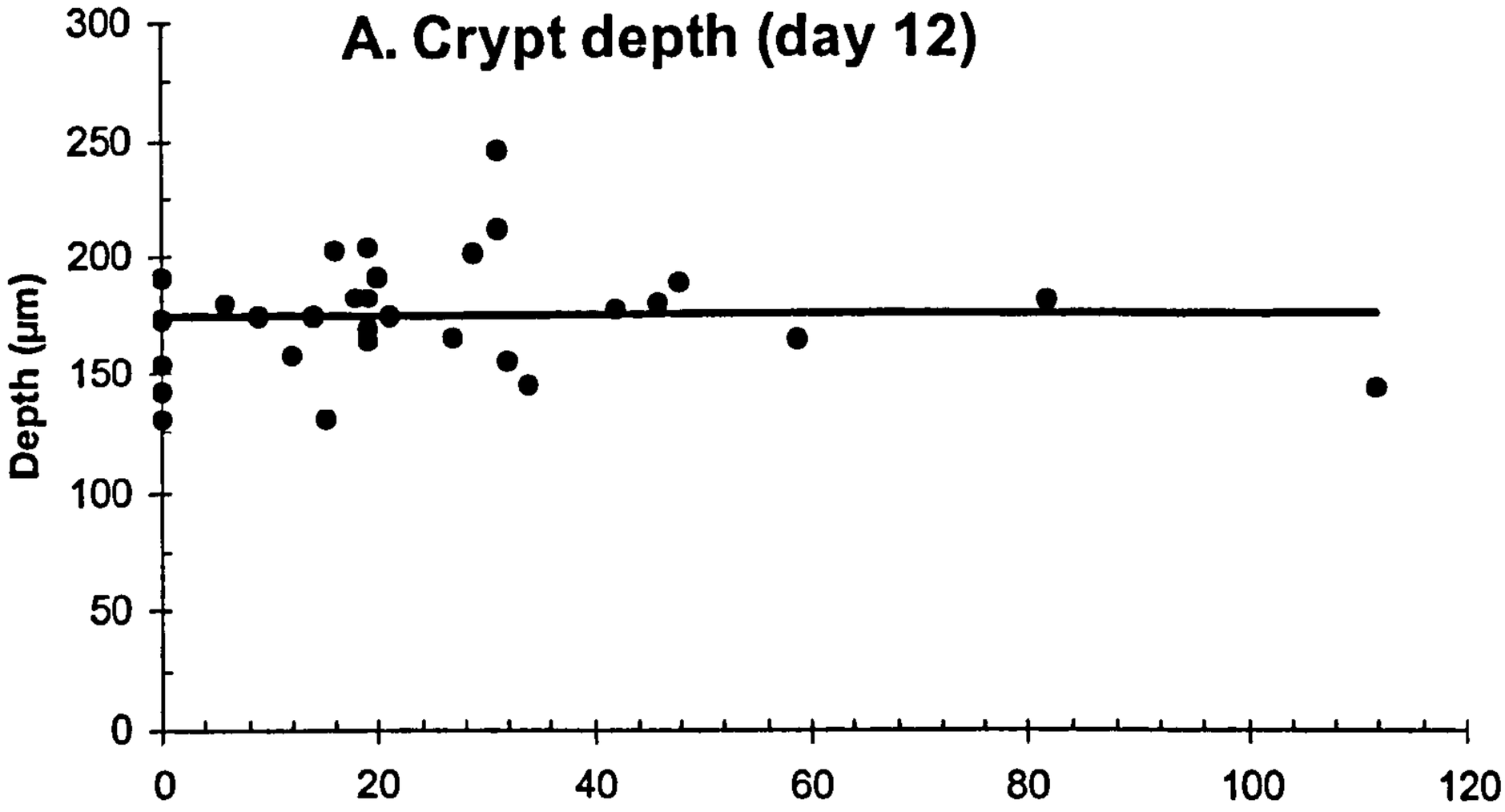
A- Crypt depth (µm) in experiment 5 (day 12).

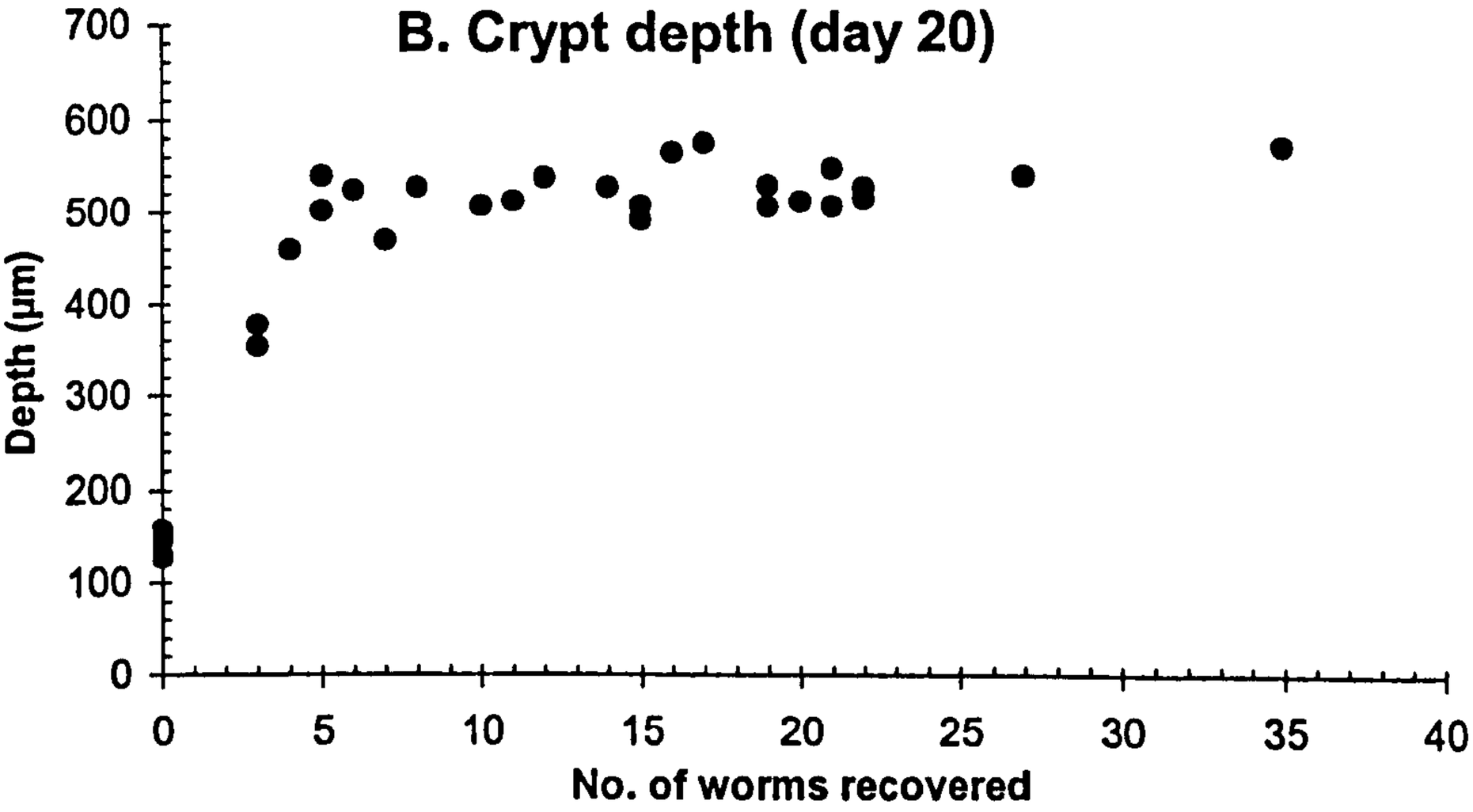
B- Crypt depth (µm) in experiment 6 (day 20).

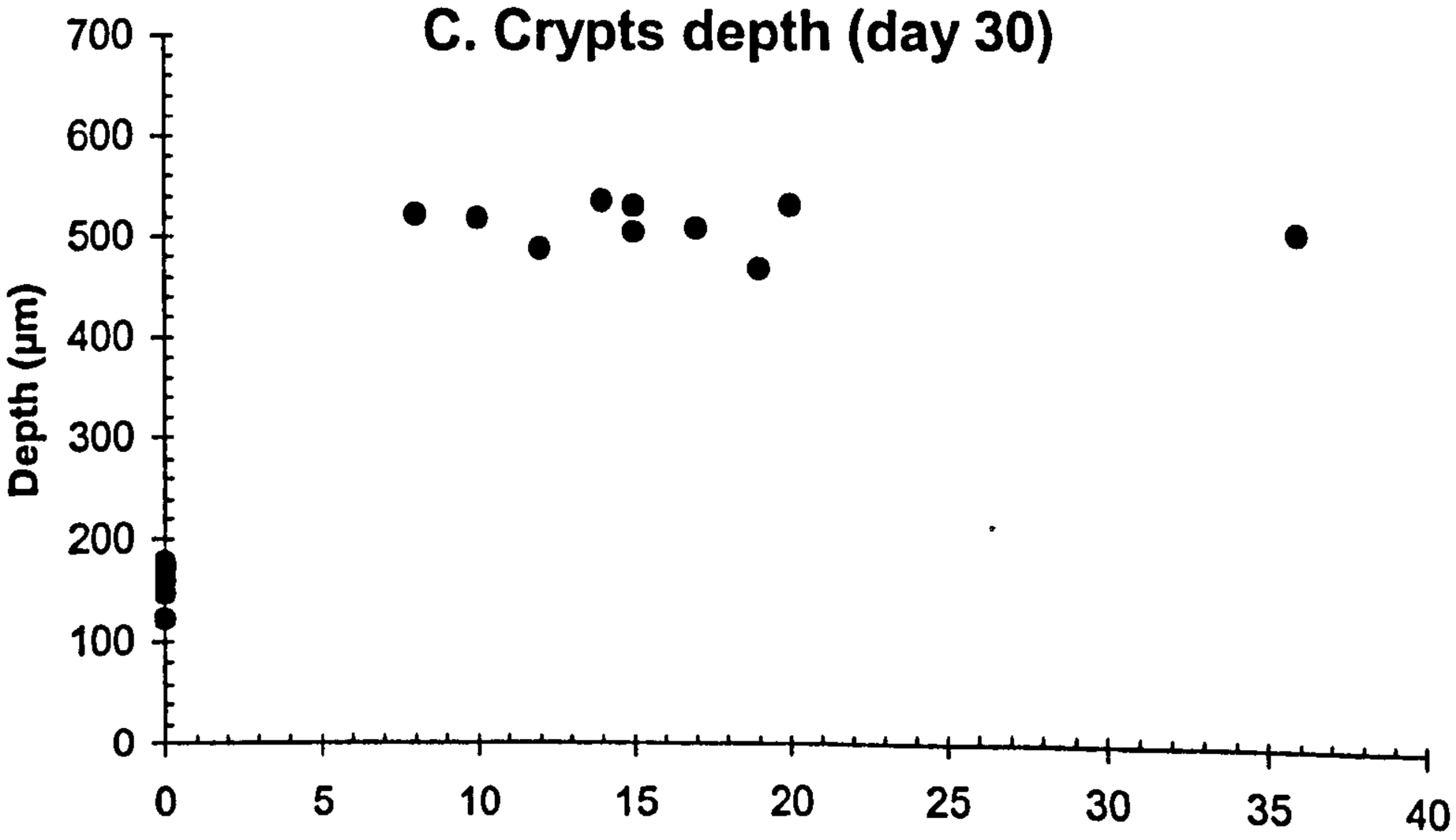
C- Crypt depth (µm) of experiment 5 (day 30).

Statistical analysis by correlation coefficient test

On day 12 PI (experiment 5)	r_s=0.183, n=30, P=0.334
On day 20 PI (experiment 6)	r_s=0.741, n=30, P<0.005
On day 30 PI (experiment 5)	r_s=0.628, n=15, P=0.012


A. Crypt depth (day 12)
Depth (µm)
300
250
200
150
100
50
0
0
20
40
60
80
100
120


B. Crypt depth (day 20)
Depth (µm)
700
600
500
400
300
200
100
0
0
5
10
15
20
25
30
35
40
No. of worms recovered


C. Crypts depth (day 30)
Depth (µm)
700
600
500
400
300
200
100
0
0
5
10
15
20
25
30
35
40

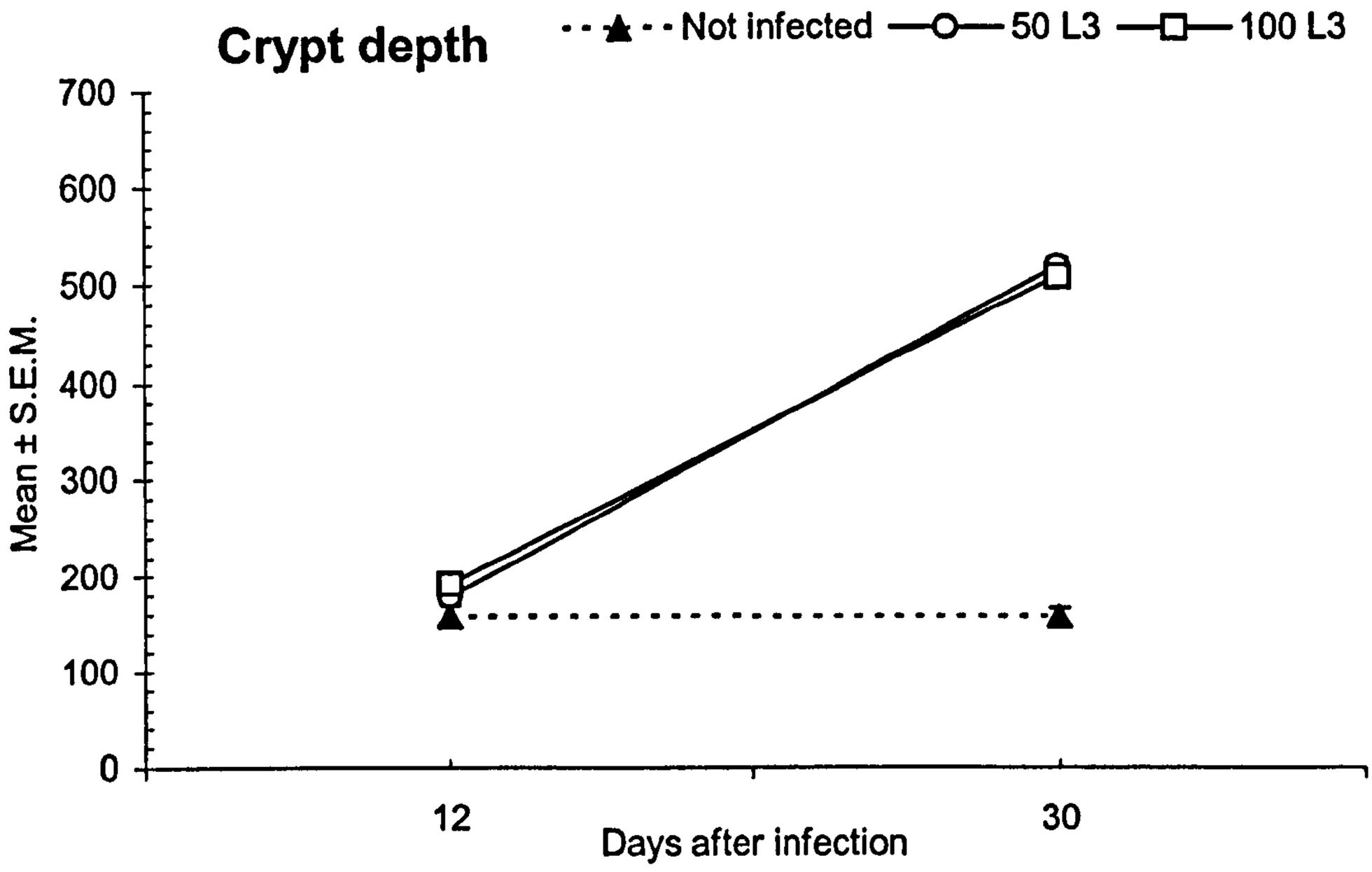


Figure 3.22 – D. Experiment 5. (Dose-response experiments). Comparison of crypt depth during primary infection at different doses of *A. ceylanicum* (Naïve, 50 L3 and 100 L3) and on two different days (12 and 30) after infection.

Statistical analysis

Data analysis of this experiment by 2-way ANOVA with time (2 levels, days 12 and 30revealed) and treatment (3 levels, Naïve, 50 and 100 L3 *A.ceylanicum*) showed:

Main effect of time ($F_{1,24}$=840.953, P<0.001)

Main effect of treatment ($F_{2,24}$=290.271, P<0.001)

Interaction between treatment and time ($F_{2,29}$=213.478, P<0.001)

Model R^2=0.985

Figure 3.23 – (Dose-response experiments). Relationship between the number of worms recovered and the number of mitotic figure observed in the crypt in hamsters infected with different doses of *A. ceylanicum* (L3).

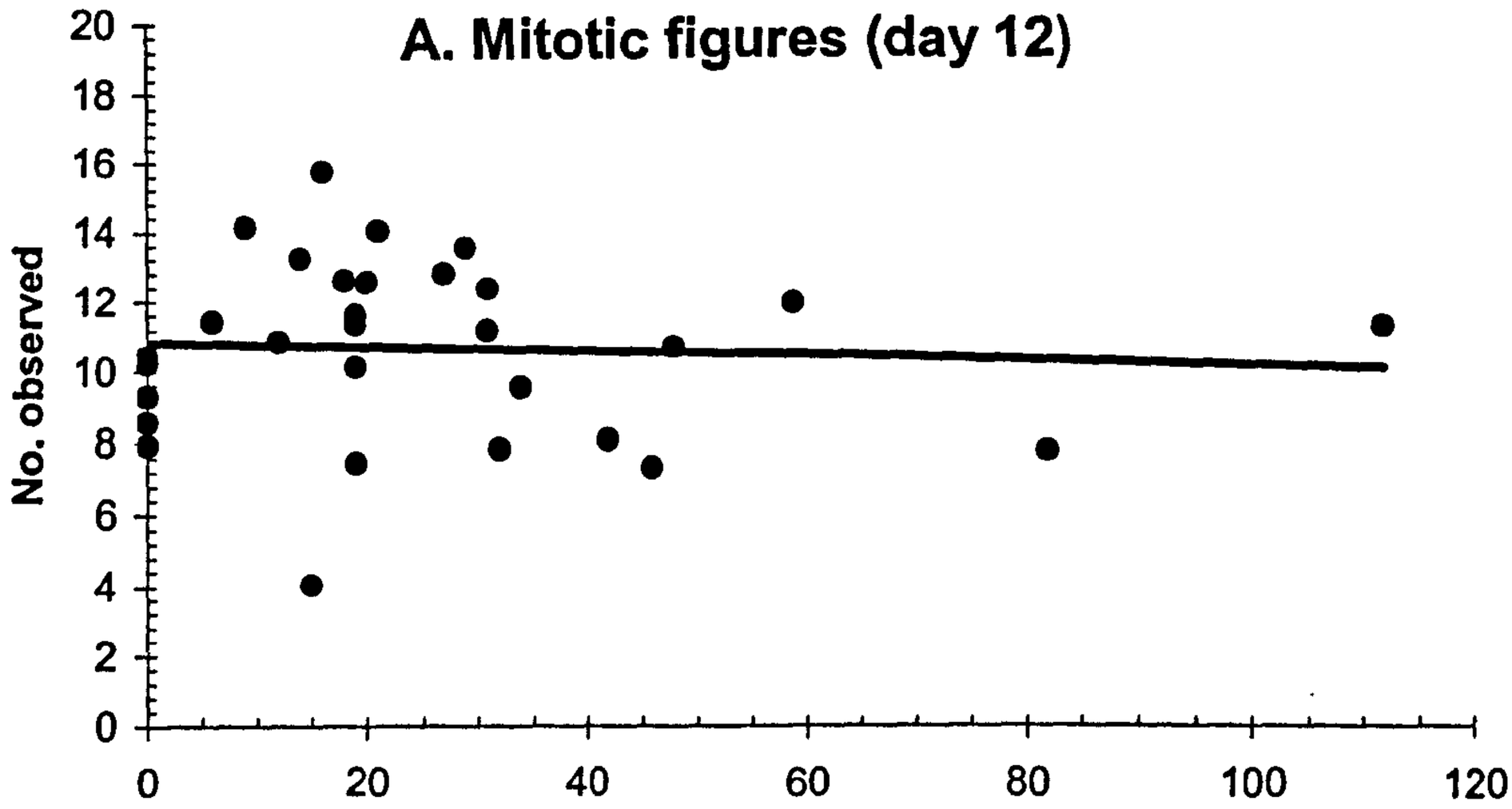
A- mitotic fig./crypt in experiment 5 (day 12).

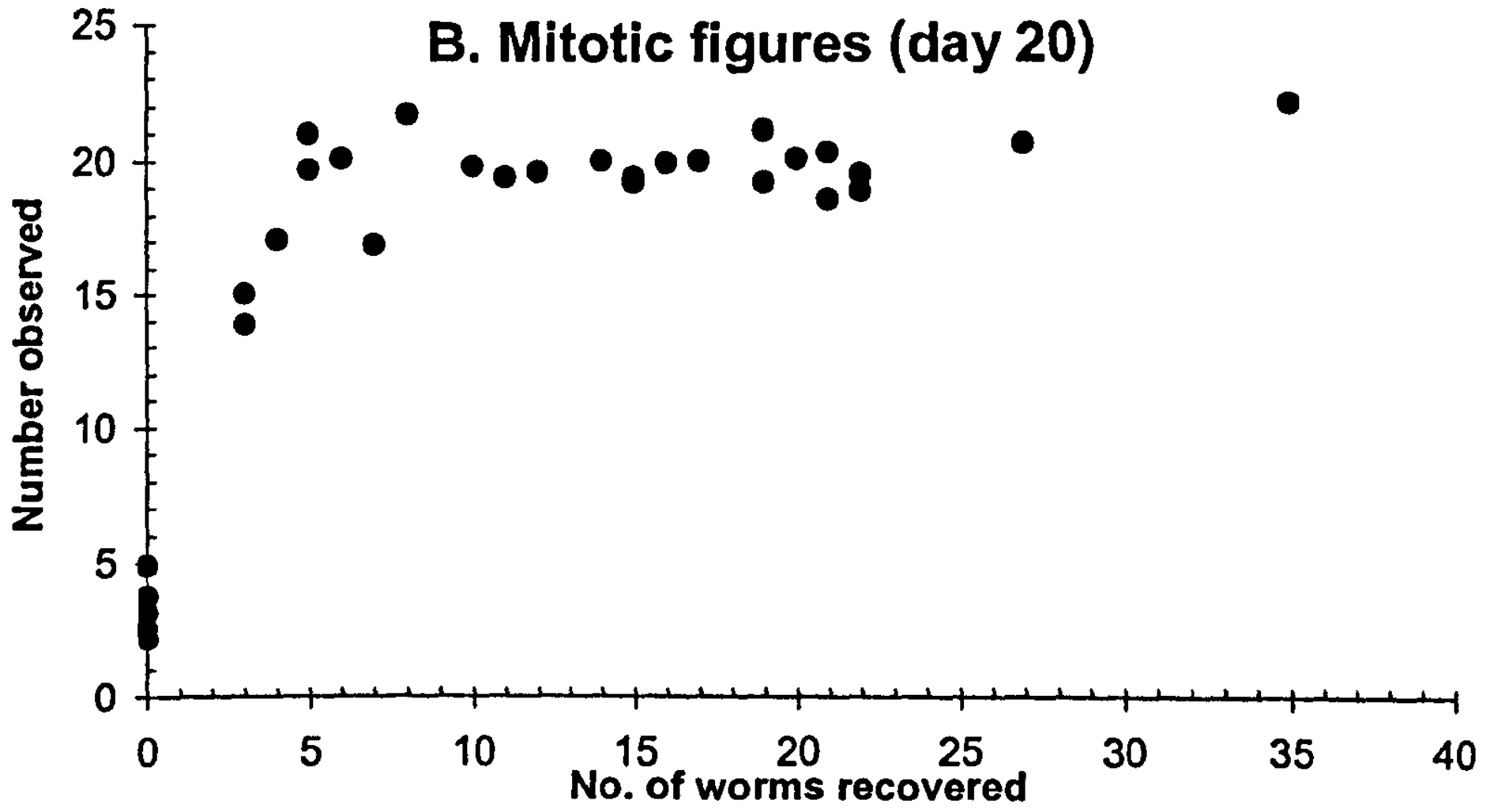
B- Mitotic fig./crypt in experiment 6 (day 20).

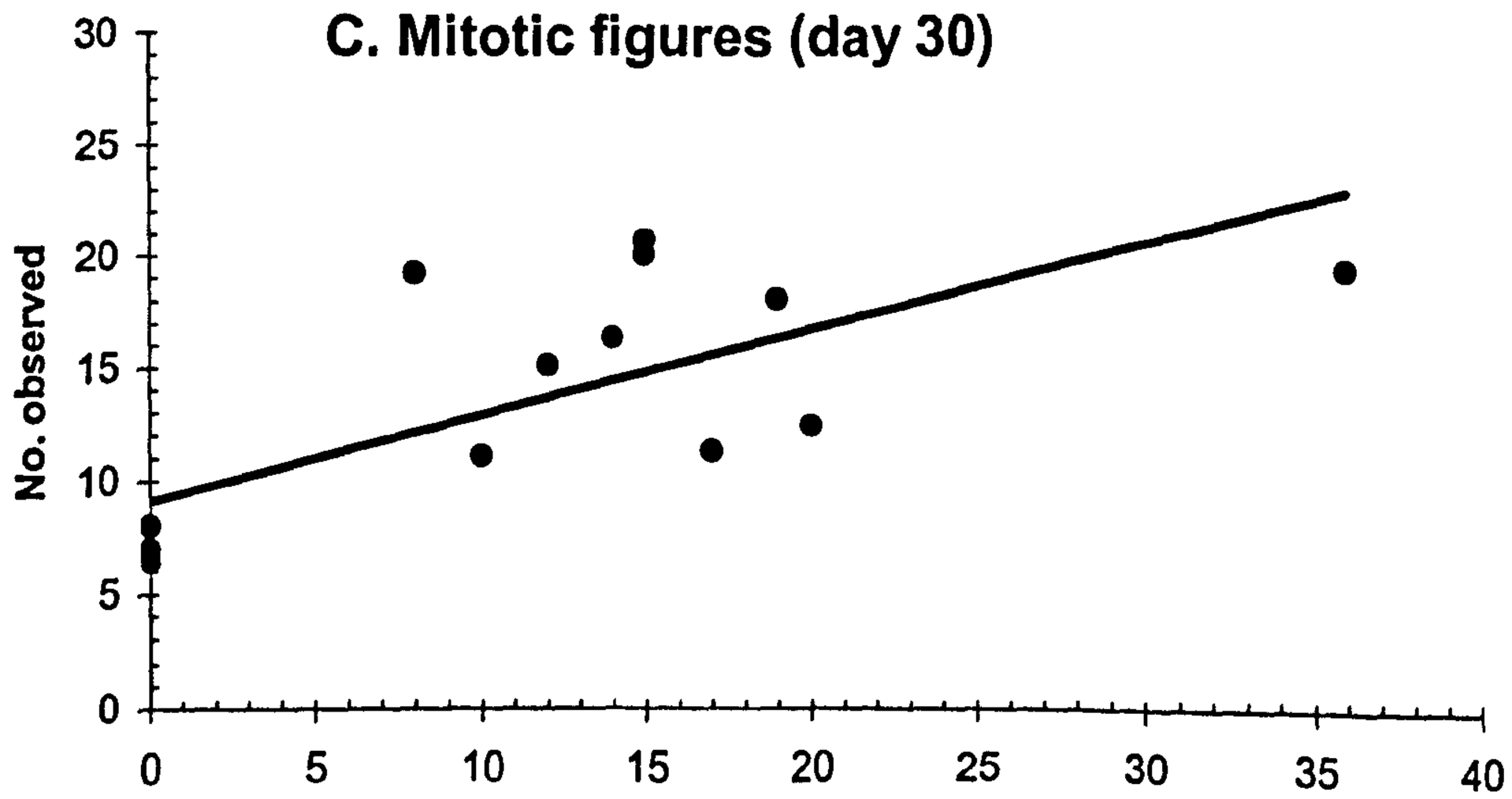
C- Mitotic fig./crypt of experiment 5 (day 30).

Statistical analysis by correlation coefficient test

On day 12 PI (experiment 5)	r_s=-0.049, n=30, P=0.798
On day 20 PI (experiment 6)	r_s=0.620, n=30, P<0.000
On day 30 PI (experiment 5)	r_s=0.727, n=15, P=0.002


A. Mitotic figures (day 12)
No. observed
0
2
4
6
8
10
12
14
16
18
20
0
20
40
60
80
100
120


B. Mitotic figures (day 20)
Number observed
0
5
10
15
20
25
0
5
10
15
20
25
30
35
40
No. of worms recovered


C. Mitotic figures (day 30)
No. observed
0
5
10
15
20
25
30
0
5
10
15
20
25
30
35
40

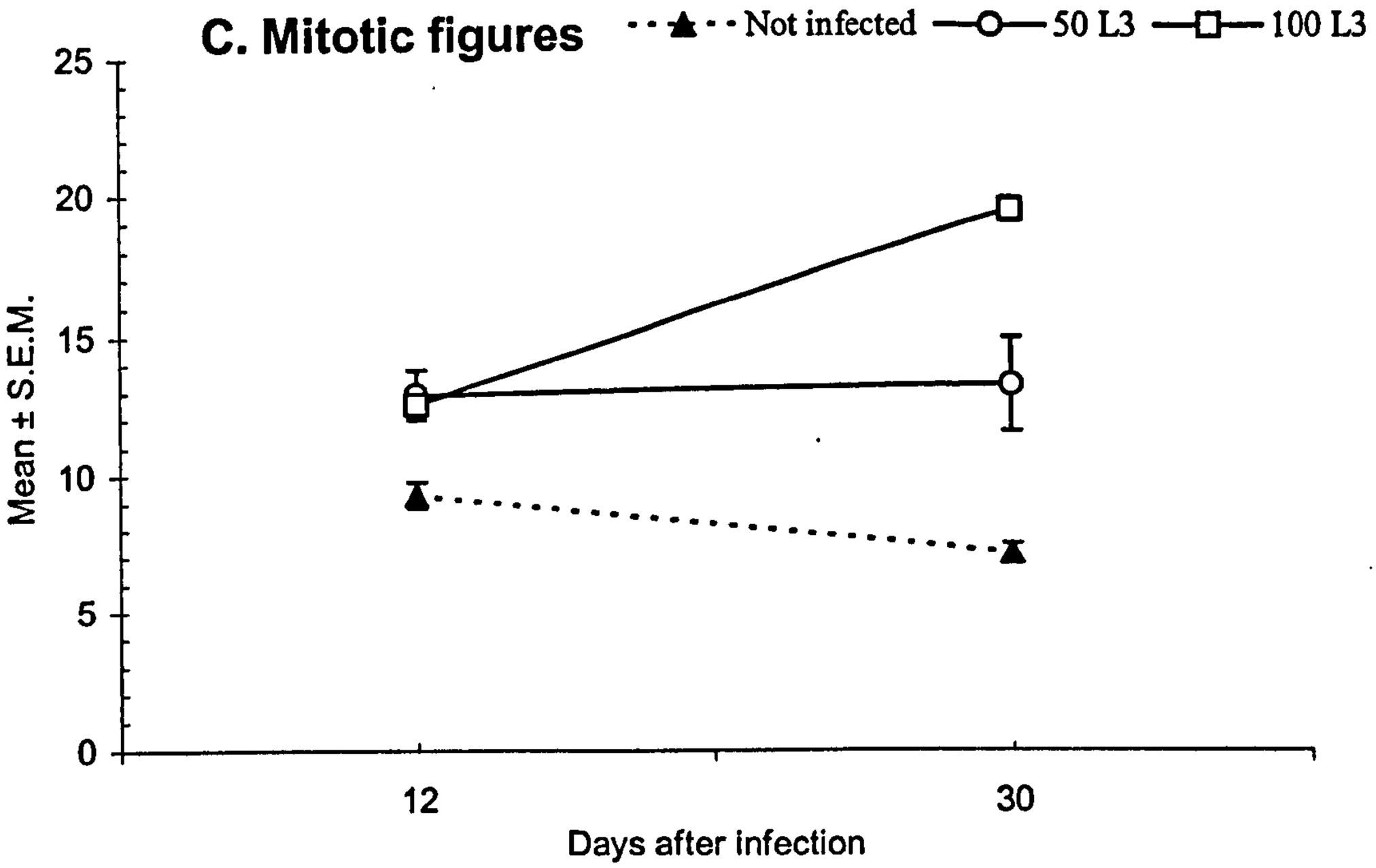


Figure 3.23 - D Experiment 5. (Dose-response experiments). Number of mitotic figure in the crypt of hamsters infected with different doses of (0, 50L3 and 100L3) *A. ceylanicum* and at two different days (12 and 30) after infection.

Statistical analysis

Data analysis of this experiment by 2-way ANOVA with time (2 levels, days 12 and 30 revealed) and treatment (3 levels, Naïve, 50 and 100 L3 *A.ceylanicum*) showed:

Main effect of time ($F_{1,24}$=9.589, P<0.01)

Main effect of treatment ($F_{2,24}$=67.864, P<0.001)

Interaction between treatment and time ($F_{2,24}$=23.751, P<0.001)

Model R^2=0.866

Figure 3.24 – (Dose-response experiments). Relationship between the number of worms recovered and the number of mast cells in (mm^2) in hamsters infected with different doses of L3 *A. ceylanicum*.

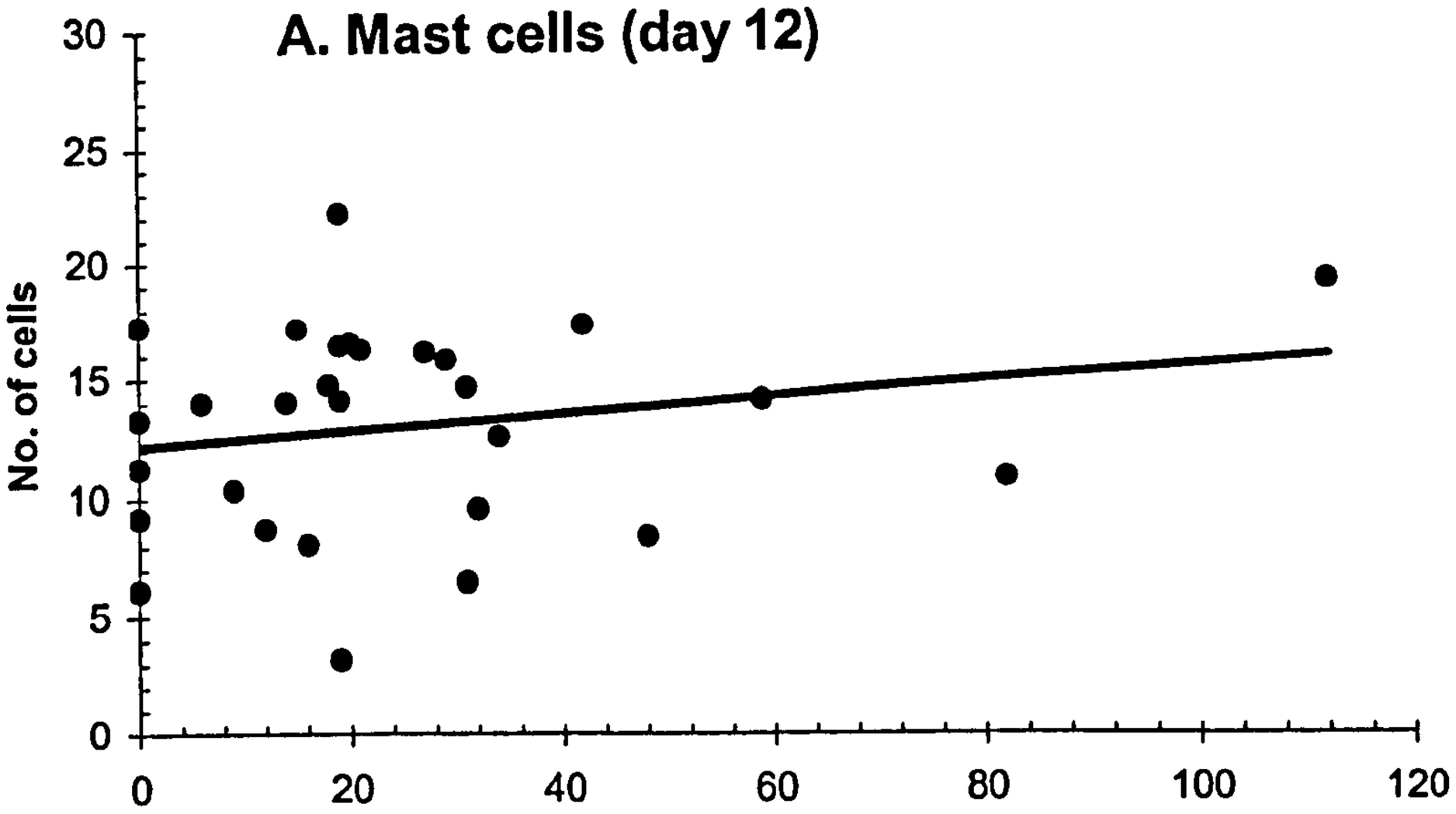
A- Mast cells/ mm^2 of experiment 5 (day 12).

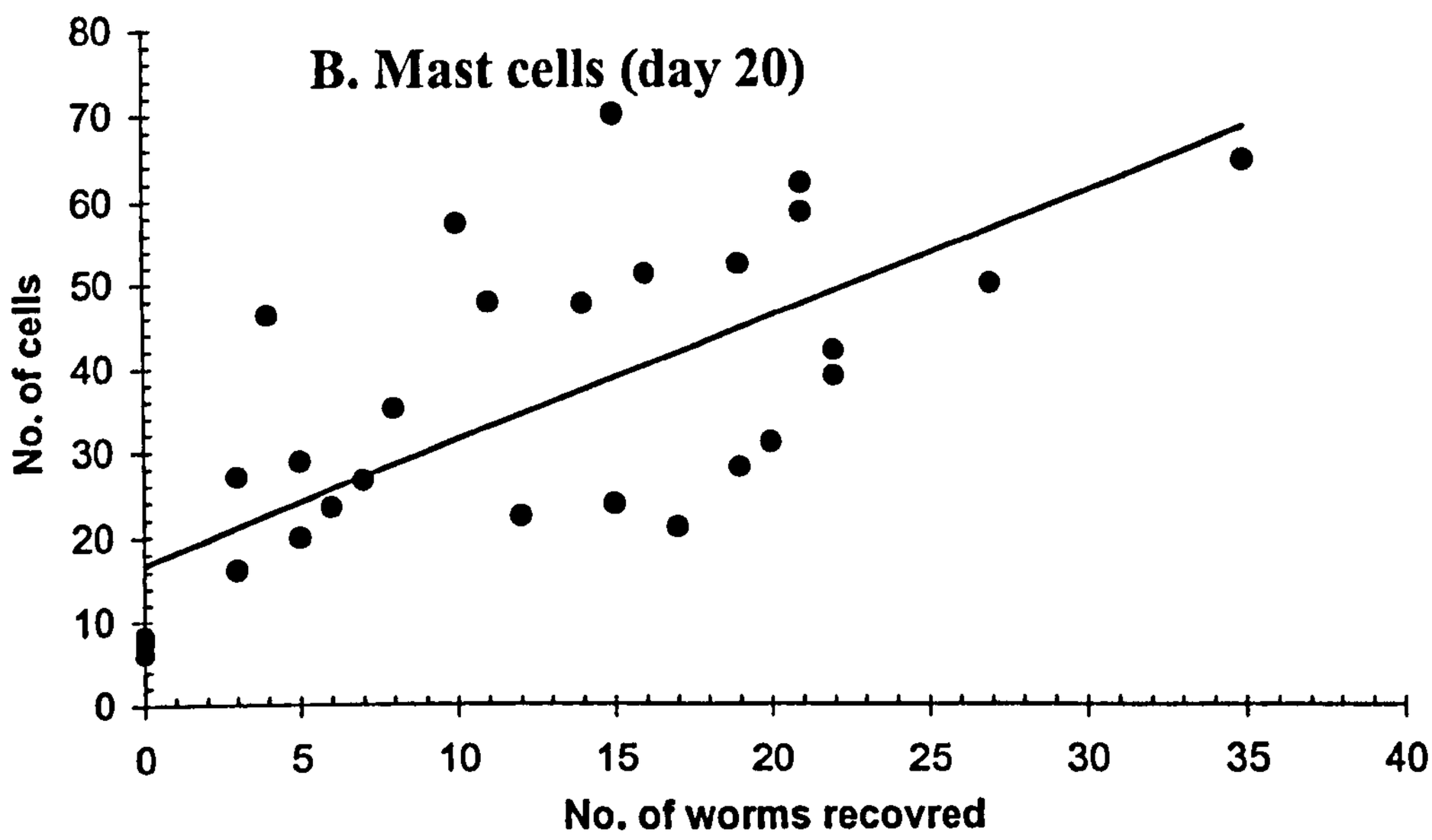
B- Mast cells/mm^2 of experiment 6 (day 20).

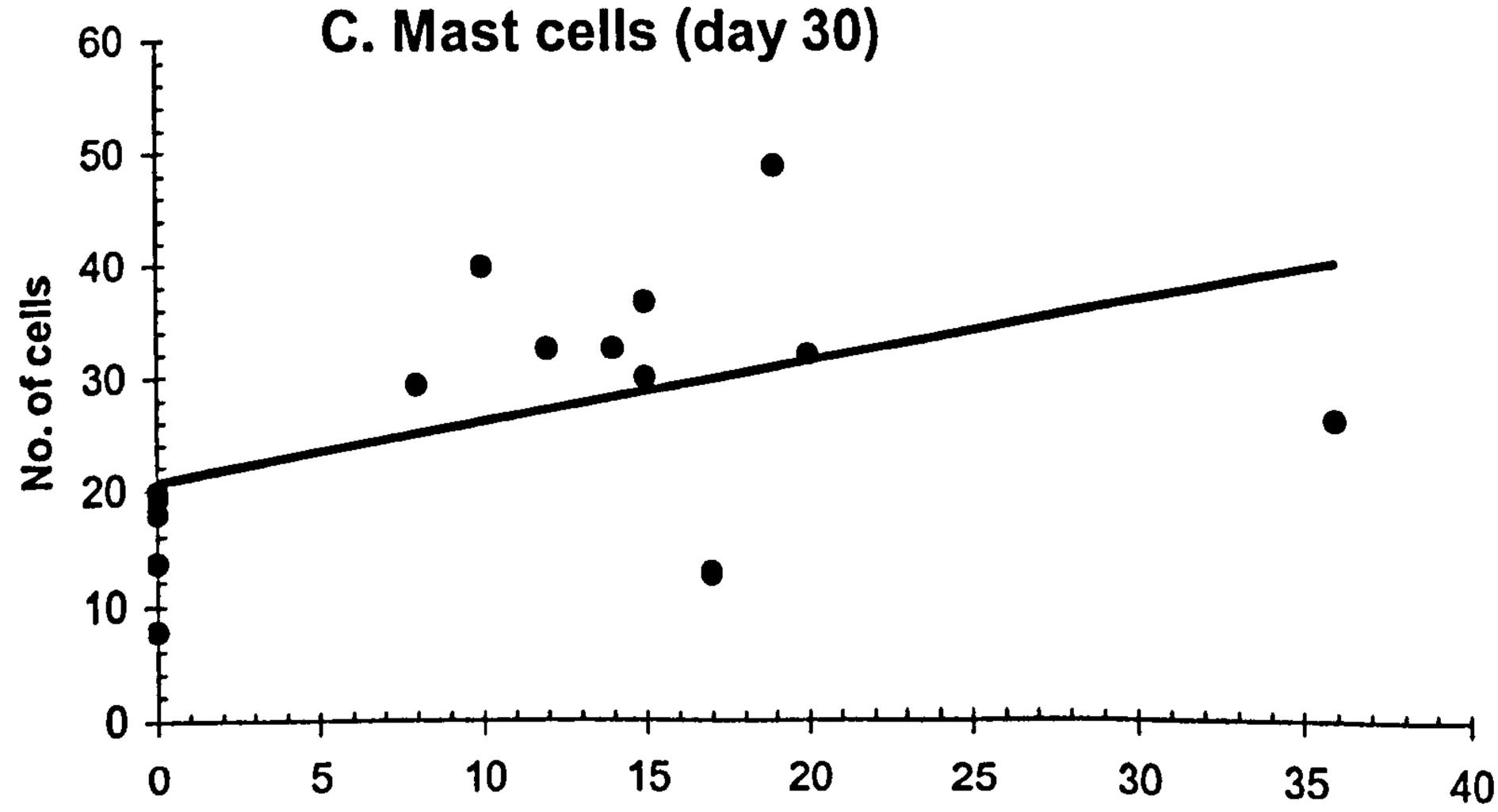
C- Mast cells/ mm^2 of experiment 5 (day 30).

Statistical analysis by correlation coefficient test

On day 12 PI (experiment 5)	r_s=0.185, n=29, P=0.336
On day 20 PI (experiment 6)	r_s=0.725, n=30, P<0.005
On day 30 PI (experiment 5)	r_s=0.484, n=15, P=0.06


A. Mast cells (day 12)
No. of cells
0
5
10
20
25
30
0
20
40
60
80
100
120


B. Mast cells (day 20)
No. of cells
0
10
20
30
40
50
60
70
80
0
5
10
15
20
25
30
35
40
No. of worms recovred


C. Mast cells (day 30)
No. of cells
0
10
20
30
40
50
60
0
5
10
15
20
25
30
35
40

cells/mm^2 of naïve uninfected hamsters to 12.4 ± 1.7 cells/mm^2 and 15.9 ± 1.9 cells/mm^2 in hamsters infected with 50L3 and 100L3 *A. ceylanicum* respectively were significant (main effect of time, $F_{1,24}$=30.944, P <0.001 and treatment, $F_{2,24}$=7.923, P <0.01). Similarly, mast cells changed significantly from the mean of 15.7 ± 2.3 cells/mm^2 in uninfected hamsters to 29.9 ± 4.5 cells/mm^2 and 34.2 ± 4.0 cells/mm^2. from treatment of 50 and 100 L3 *A. ceylanicum*. Moreover, the interaction between time and treatment (Figure 3.24 – D) was significant ($F_{2,24}$=3.584, P<0.05).

3.3.3.5 – Goblet cell responses

As can be seen from Figure 3.25 - A, the correlation between the number of worms and the number of goblet cells in Experiment 5 was not significant on day 12 (r_s=-0.055, n=29, P=0.777). However, on day 30 (Figure 3.25 - C) there was a highly significant change in goblet cells with increasing worm burden (r_s=0.799 n=14, P=0.001). A similar correlation between goblet cells and number of worms recovered was found to be significant (r_s=-0.948, n=30, P=0.001) on day 20 of Experiment 6 (Figure 3.25 - B). 2-way ANOVA from Experiment 5 showed that the interaction between time and treatment was significant ($F_{2,22}$=12.406, P<0.05) and more further, there were highly significant main effects of time ($F_{1,22}$=33.212, P<0.001) and treatment ($F_{2,22}$=11.300, P<0.001). Figure 3.25 - D shows the mean number of goblet cells, which increased from 63.6 ± 8.3 in groups that received no infection to 262.6 ± 21.5 and 179.95 ± 39.3 cells. of groups received 50L3 and 100 L3 respectively, on day 30 was significant. However, no differences between treatments were detected on day 12.

3.3.3.6 – Paneth cell responses

Correlations between the number of Paneth cells in the Crypt of hamsters' intestines and the number of worms recovered were analysed by Spearman's rank order test. The results from Experiment 5 are presented in figure 3.26 - A,

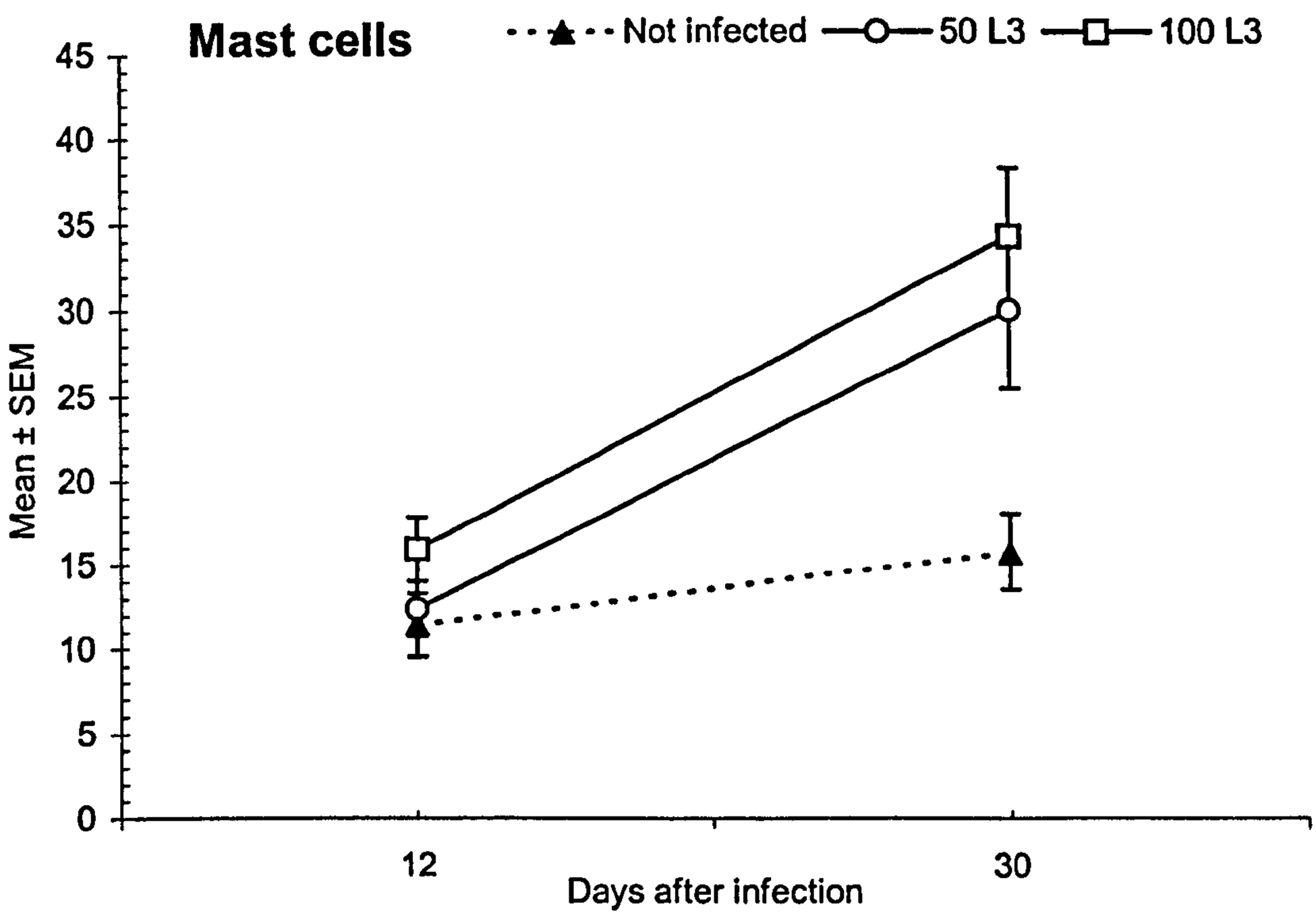


Figure 3.24 - D Experiment 5. (Dose-response experiments). Number of mast cells in hamsters infected with two different doses of *A. ceylanicum* (50 L3 and 100L3) and at two different days (12 and 30) after infection.

Statistical analysis

Data analysis of this experiment by 2-way ANOVA with time (2 levels, days 12 and 30 revealed) and treatment (3 levels, naïve, 50 and 100 L3 *A.ceylanicum*) showed:

Main effect of time ($F_{1,24}$=30.944, P<0.01)

Main effect of treatment ($F_{2,24}$=7.923, P<0.001)

Interaction between treatment and time ($F_{2,24}$=3.583, P<0.05)

Model R^2=0.628

Figure 3.25 – (Dose-response experiments). Relationship between the number of worms recovered and the number of goblet cells in (mm^2) in hamsters infected with different doses of L3 *A. ceylanicum*.

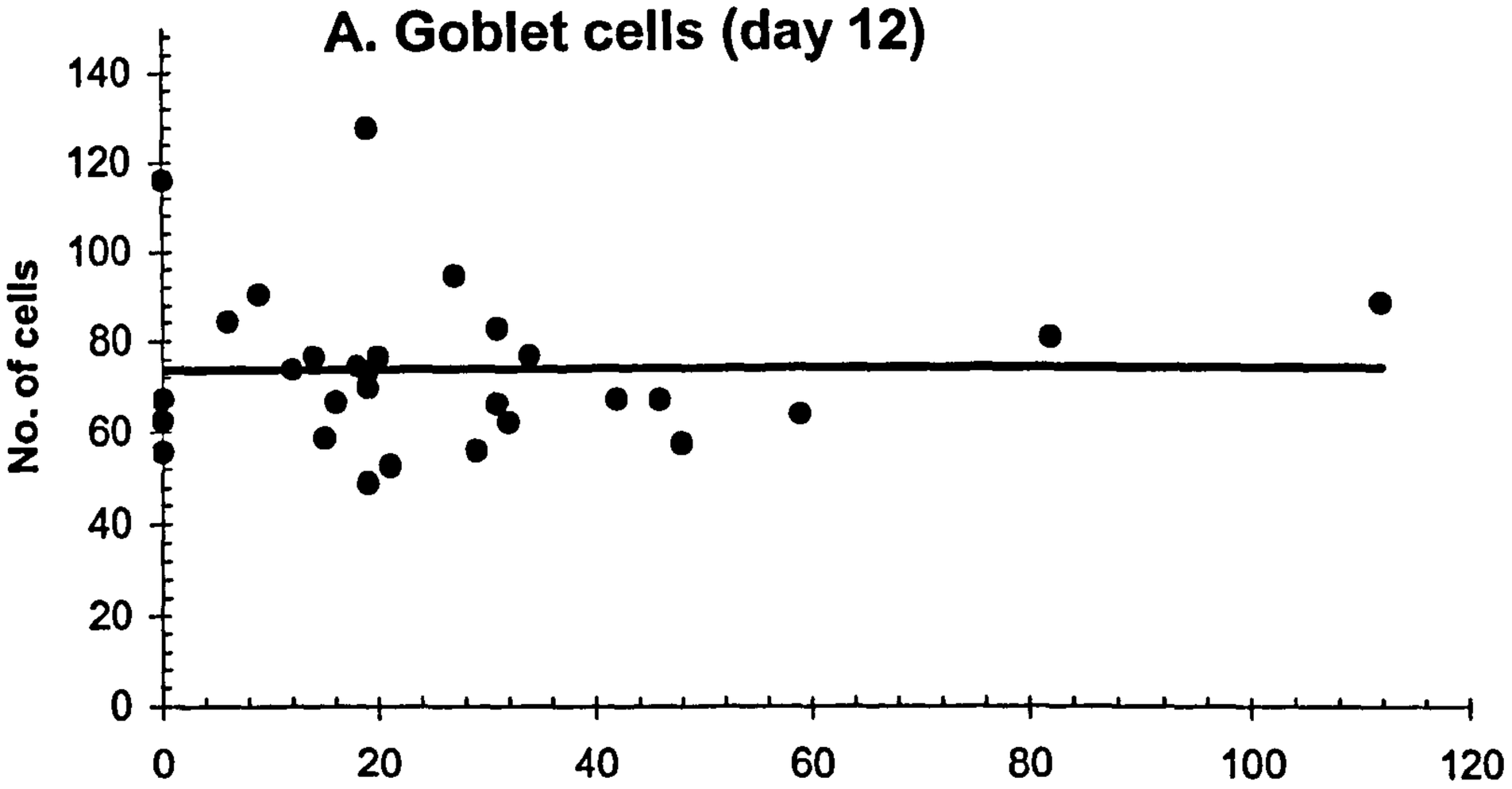
A- Goblet cells/ mm^2 of experiment 5 (day 12).

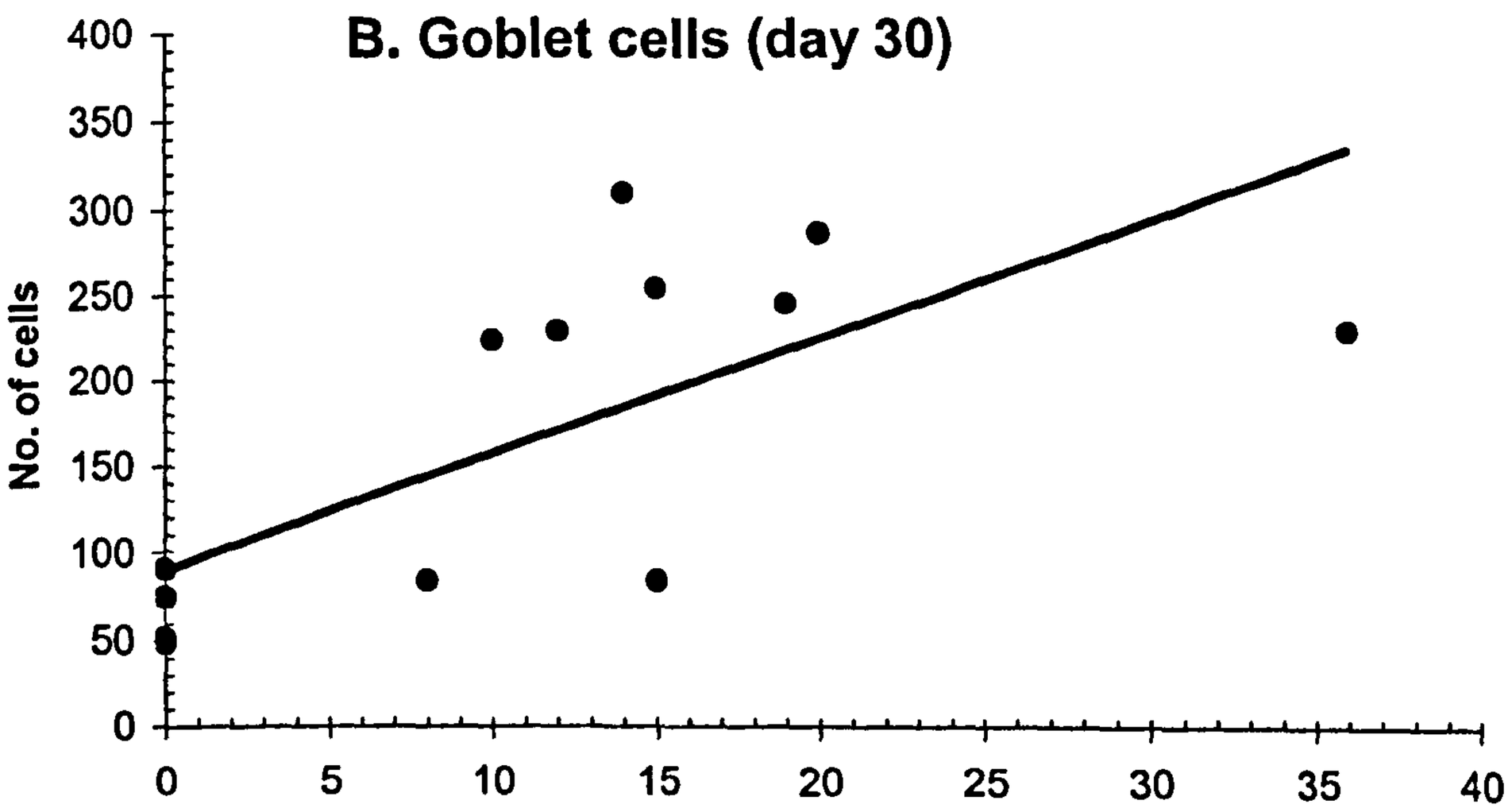
B- Goblet cells/ mm^2 of experiment 6 (day 20).

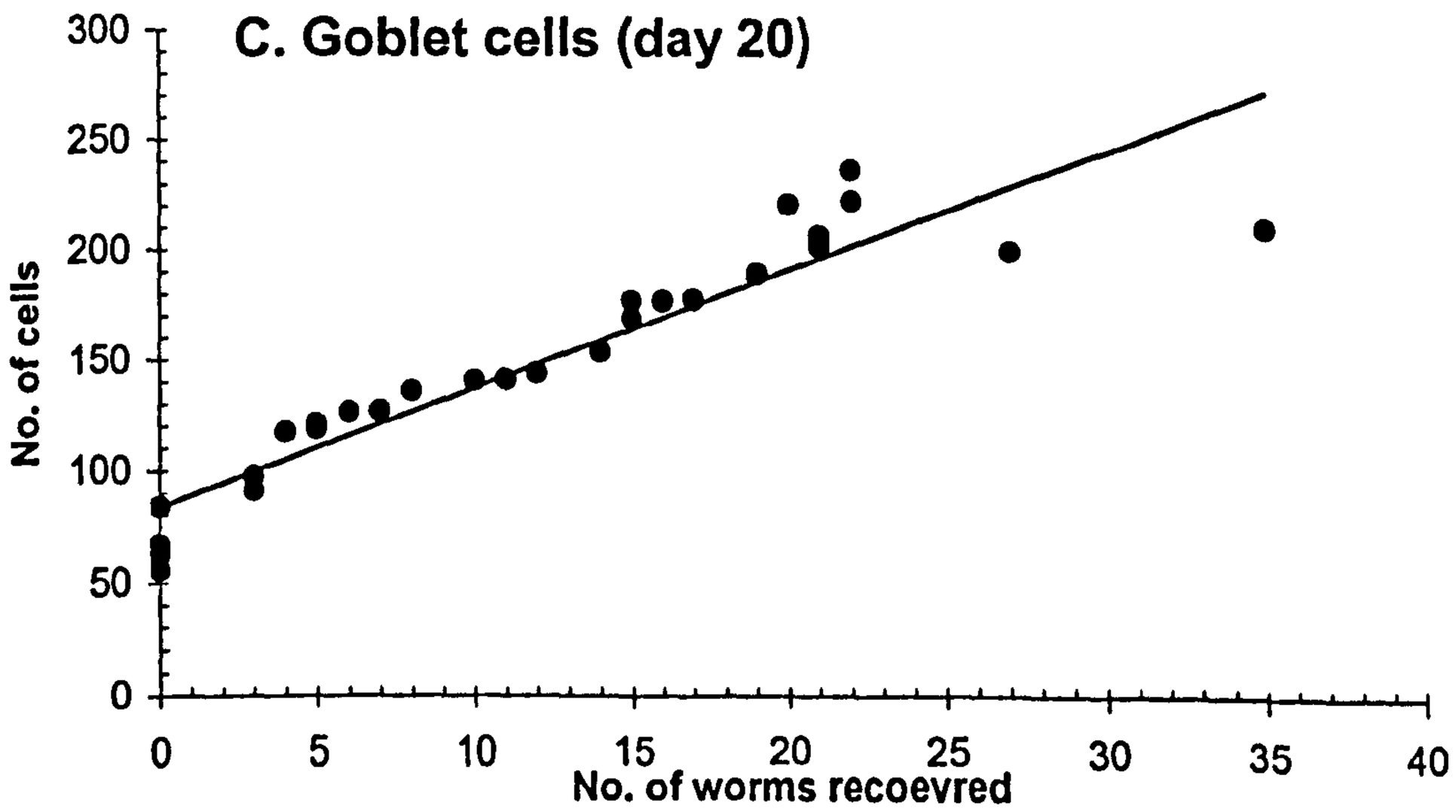
C- Goblet cells/ mm^2 of experiment 5 (day 30).

Statistical analysis by correlation coefficient test

On day 12 PI (experiment 5)	r_s=-0.055, n=29, P=0.777
On day 20 PI (experiment 6)	r_s=-0.948, n=30, P<0.001
On day 30 PI (experiment 5)	r_s=0.799 n=14, P<0.001


A. Goblet cells (day 12)
No. of cells
140
120
100
80
60
40
20
0
0
20
40
60
80
100
120


B. Goblet cells (day 30)
No. of cells
400
350
300
250
200
150
100
50
0
0
5
10
15
20
25
30
35
40


C. Goblet cells (day 20)
No. of cells
300
250
200
150
100
50
0
0
5
10
15
20
25
30
35
40
No. of worms recoevred

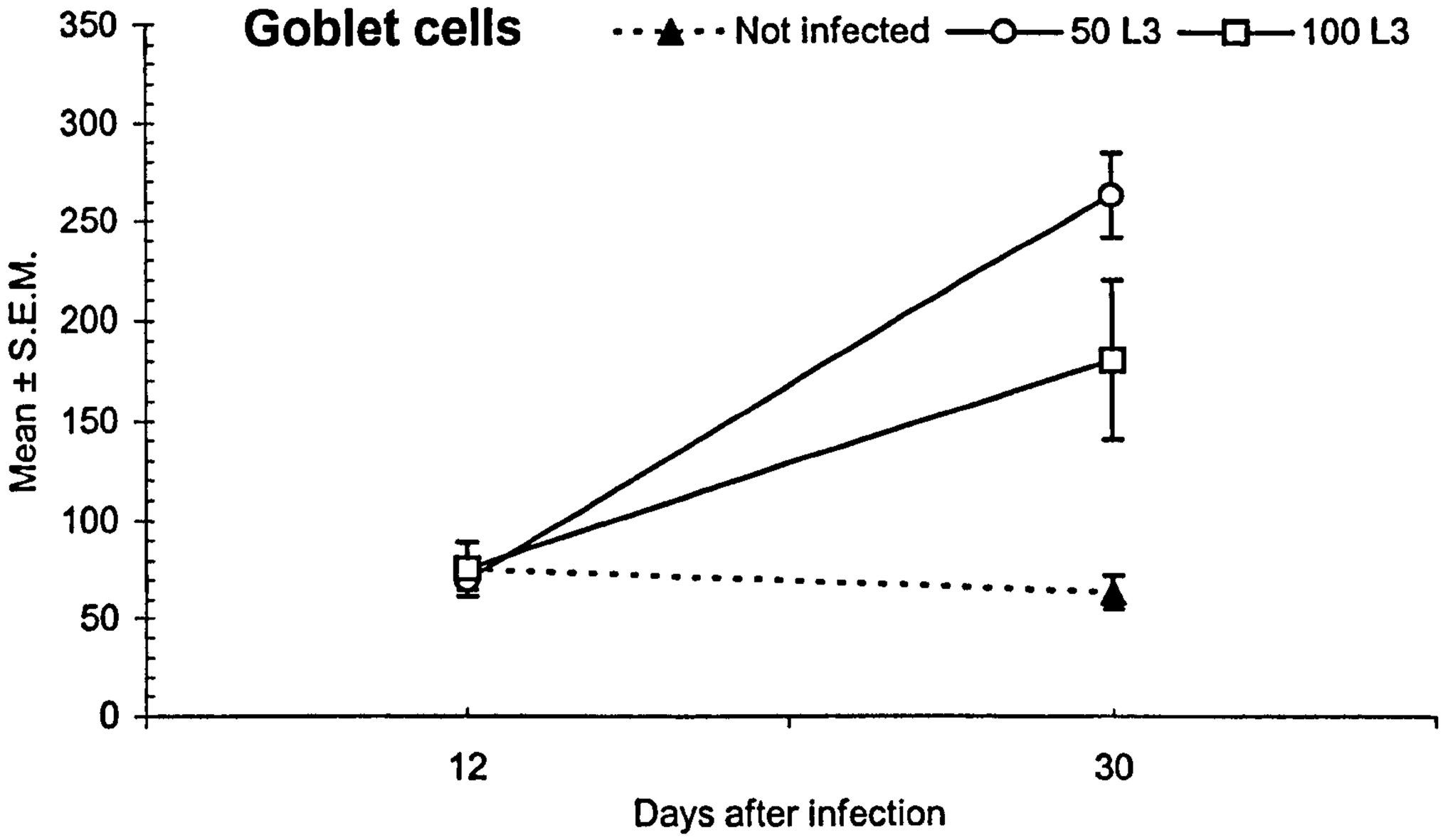


Figure 3.25 - D Experiment 5. (Dose-response experiments). Relationship between number of Goblet cells in the intestine of hamsters and the number of worms recovered at two different times (day 12 and 30)

Statistical analysis

Data analysis of this experiment by 2-way ANOVA with time (2 levels, days 12 and 30revealed) and treatment (3 levels, Naïve, 50 and 100 L3 *A.ceylanicum*) showed:

Main effect of time ($F_{1,22}$=33.212, P<0.001)

Main effect of treatment ($F_{2,22}$=11.300, P<0.001)

Interaction between treatment and time ($F_{2,22}$=12.406, P<0.05)

Model R^2=0.728

and there show that there were no significant differences on day 12 (r_s=-0.317, n=30, P=0.0876), however, figure 3.26 - C shows that in the same experiment on day 30 there was a significant negative relationship between changes in the number of Paneth cells and worms recovered at autopsy (r_s=-0.546 n=15, P=0.035). However, this change was not significant on day 20 (Fig 3.26 – B) of Experiment 6 (r_s=-0.256 n=30, P=0.086). To compare the dose response on two different days, 2-way ANOVA was applied to the data from Experiment 5 and the results illustrated in Fig. 3.26 – D show that neither the interaction between time and treatment nor the main effect of treatment affected the number of Paneth cells significantly ($F_{2,29}$=1.682, P=0.207 and $F_{2,24}$=1.167, P=0.328, respectively). However, there was significant effect of time ($F_{1,24}$=11.764, P<0.01). This figure suggested a dip in Paneth cell numbers in low level infection and a rise when worm burdens were greater than 15 worms.

3.3.3.7 – Eosinophil responses

Eosinophils were counted only in Experiment 6 and the results are presented in figure 3.27. Changes in eosinophil numbers were significant with increasing worm burdens (r_s=0.433 n=29, P<0.01). An increase was noted in eosinophil numbers in the intestines of hamsters in all groups tested compared with naïve uninfected groups.

3.3.4 – Recovery of the intestinal pathology following worm removal.

In Experiment 7 sixty adult female age-matched hamsters were used, 20 of which were infected with 50 L3 *A.ceylanicum* on day 0 then treated with Ivermectin (Anthelmintic drug) on day 28 and killed on days 35, 42, 49 and 63 pi. Another twenty-five hamsters were given an oral primary infection of 50 L3 *A.ceylanicum* and killed on days 28, 35, 42, 49 and 63 after infection. The remaining 15 hamsters were left uninfected and killed on days 28, 49 and 63. Faecal egg counts were carried out on groups A, G, E and C six day before and

Figure 3.26 – (Dose-response experiments). Relationship between Paneth cell numbers in the crypt of hamsters and the number of worms recovered following infection with different doses of L3 *A. ceylanicum*.

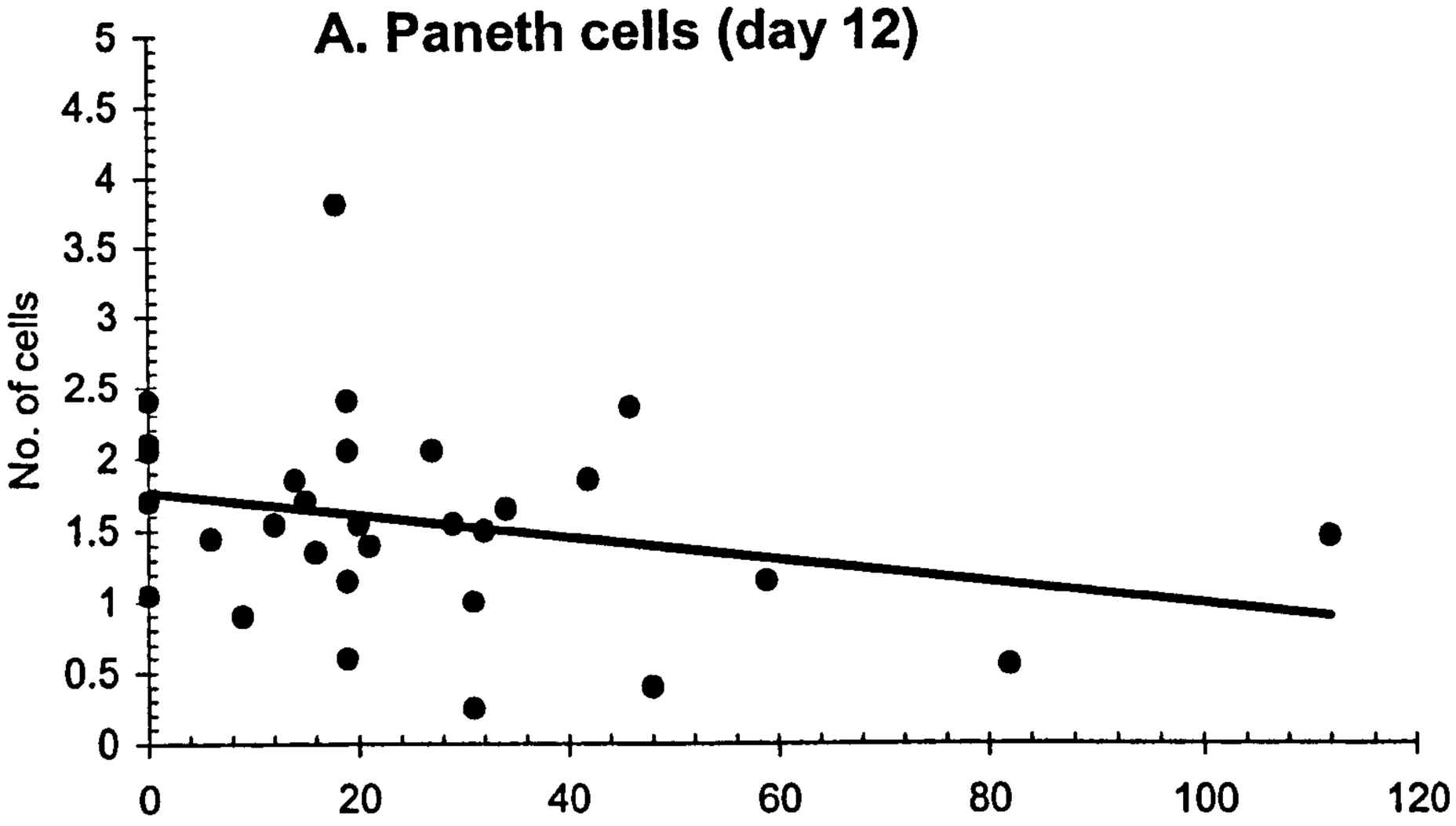
A- Paneth cells/ crypt of experiment 5 (day 12).

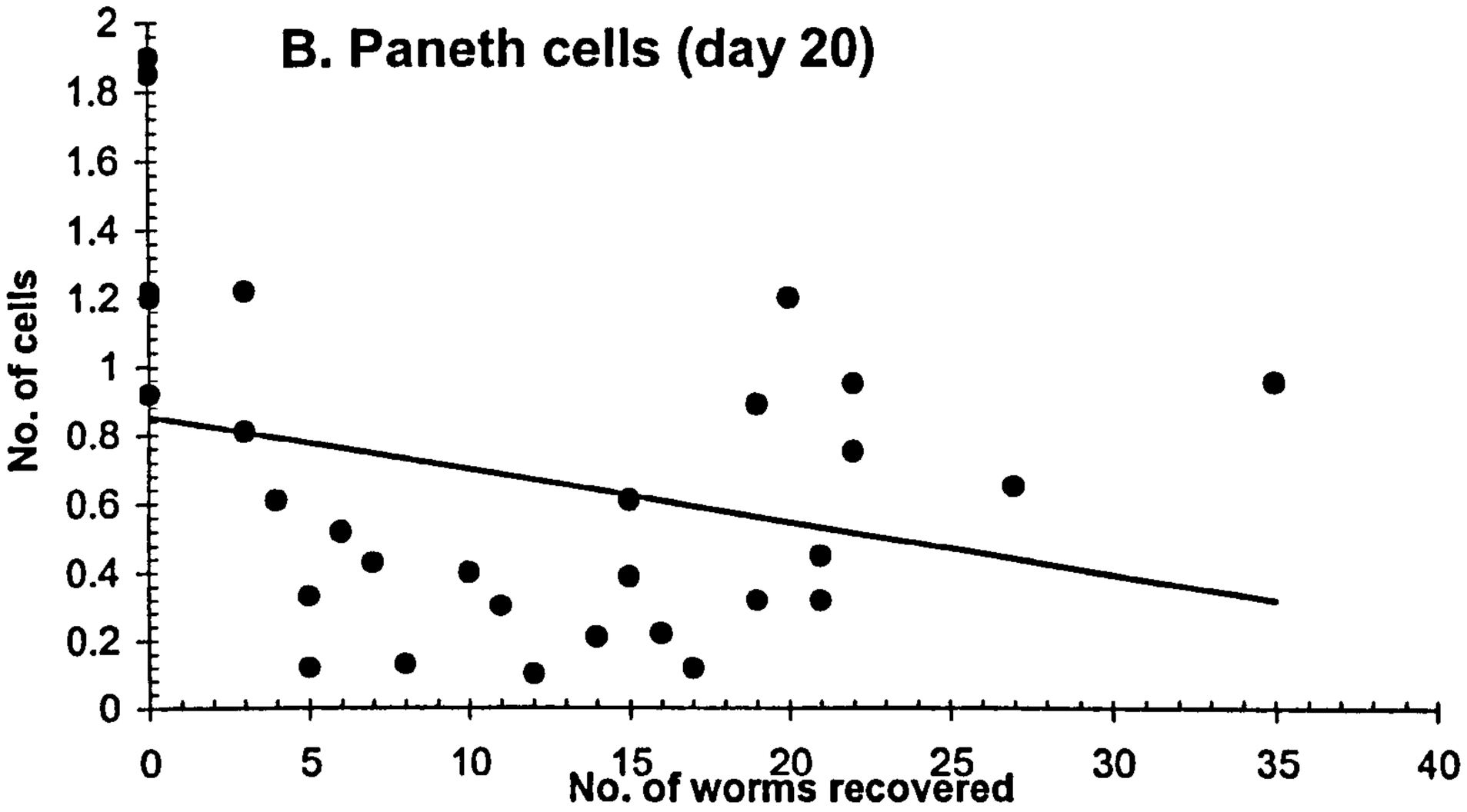
B- Paneth cells/ crypt of experiment 6 (day 20).

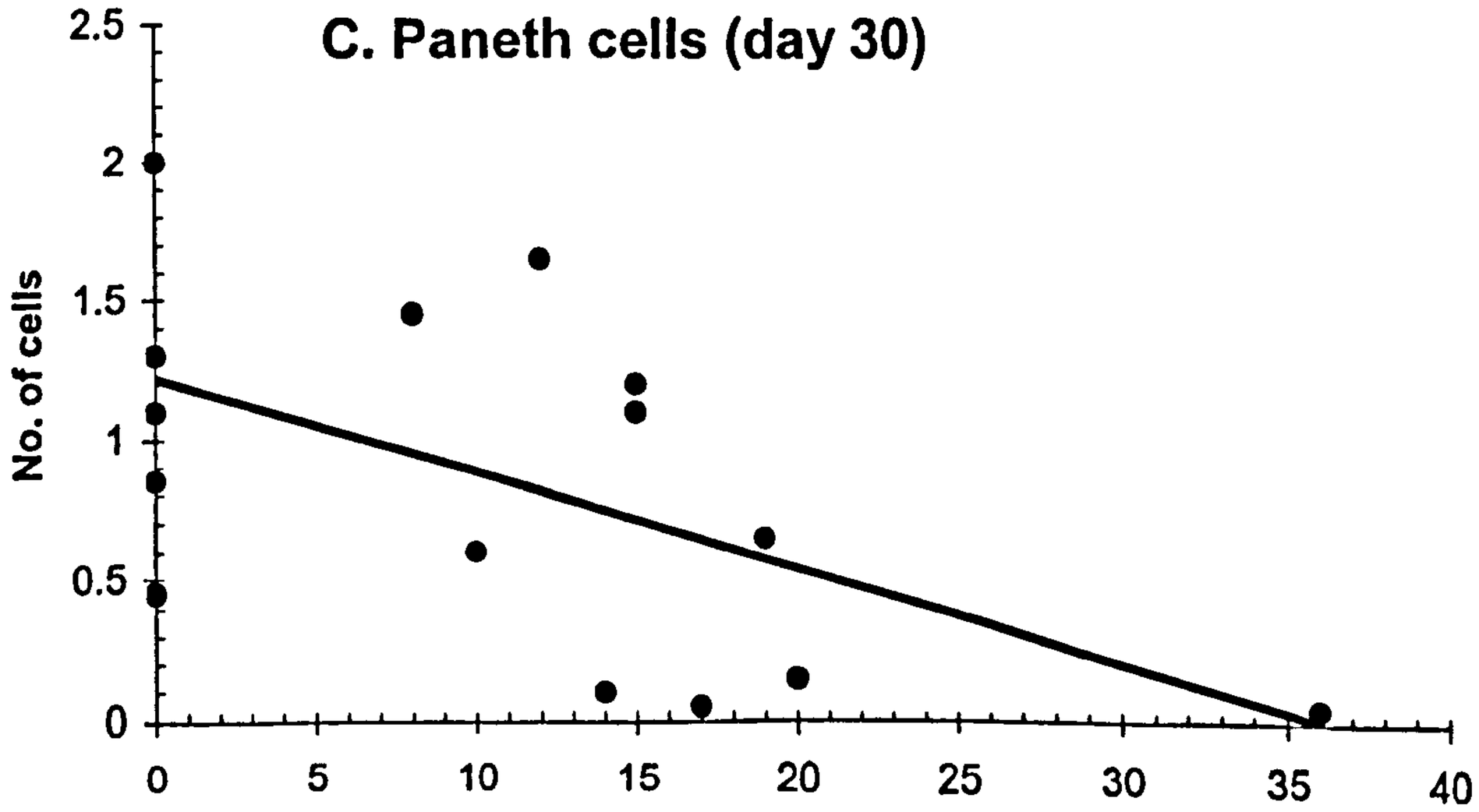
C- Paneth cells/ crypt of experiment 5 (day 30).

Statistical analysis by correlation coefficient test

On day 12 PI (experiment 5)	r_s=-0.317, n=30, P=0.0876
On day 20 PI (experiment 6)	r_s=-0.256 n=30, P=0.086
On day 30 PI (experiment 5)	r_s=-0.546 n=15, P=0.035


A. Paneth cells (day 12)
No. of cells
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0
20
40
60
80
100
120


B. Paneth cells (day 20)
No. of cells
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
5
10
15
20
25
30
35
40
No. of worms recovered


C. Paneth cells (day 30)
No. of cells
2.5
2
1.5
1
0.5
0
0
5
10
15
20
25
30
35
40

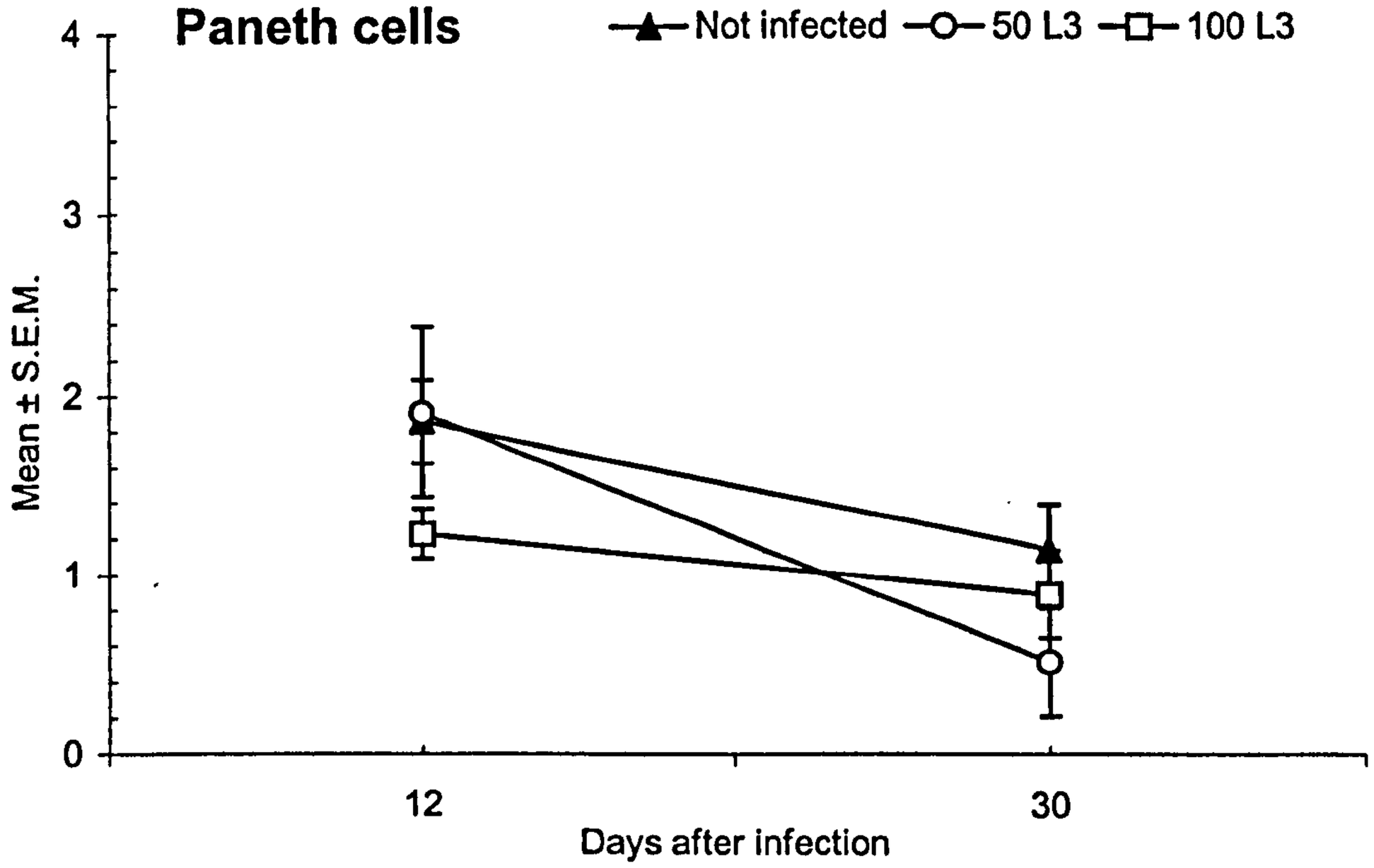


Figure 3.26 – D. Experiment 5. (Dose-response experiments). Paneth cells in the crypts of hamsters after infection with different doses of *A.ceylanicum* and on two different days (12 and 30) after infection.

Statistical analysis

Data analysis of this experiment by 2-way ANOVA with time (2 levels, days 12 and 30revealed) and treatment (3 levels, Naïve, 50 and 100 L3 *A.ceylanicum*) showed:

Main effect of time	($F_{1,24}$=11.764, P<0.01)
Main effect of treatment	($F_{2,24}$=1.167, P=0.328)
Interaction between treatment and time	($F_{2,24}$=1.682, P=0.207)

Model R^2=0.301

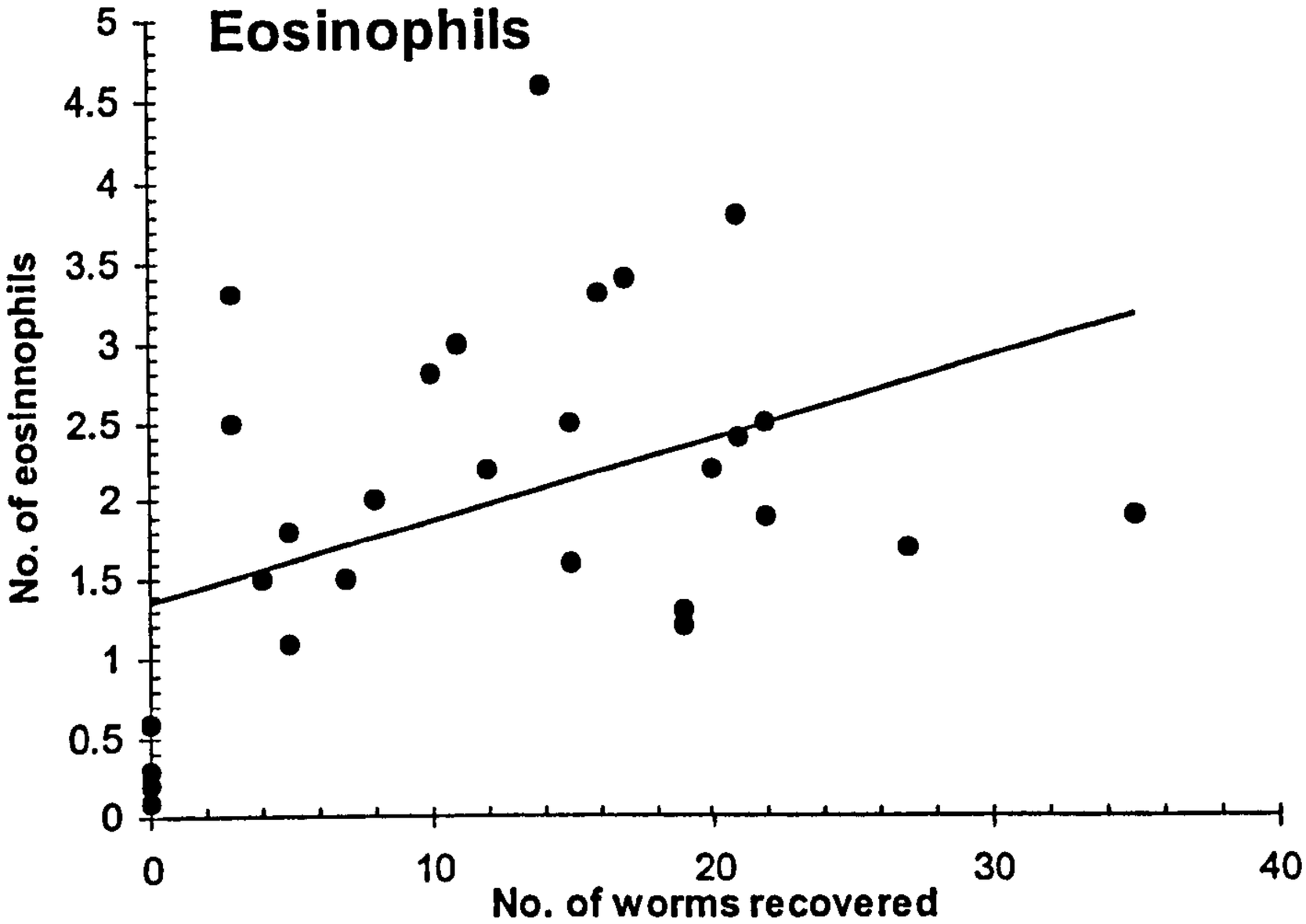


Figure 3.27 - Experiment 6. (Dose-response experiments). Changes in eosinophils in hamsters infected with six different doses of *A. ceylanicum* on day 20 PI.

Statistical analysis by correlation coefficient test

On day 20 PI (experiment 5) r_s=0.433 n=29, P=0.008

3 days after Ivermectin administration to confirm worm establishment and expulsion by the anthelmintic treatment. The design of this experiment is detailed in Table 3.7.

3.3.4.1 – Faecal egg counts

As can be seen from Figure 3.28 the number of eggs per gram of faeces declined from a range between 2000-5000 epg to no eggs at all on the day following administration of Ivermectin. This confirmed that worms originally had established in all the groups and that the anthelmintic was effective in removing the worms.

3.3.4.2 – Worm recovery

Despite the low number of worms recovered from primary infected hamsters on day 28, the usual decline in the number of worms from around 20 worms on day 35 to about 10 worms on day 63 was observed (Figure 3.29). No worms were found in the other groups, which received anthelmintic drug (Ivermectin).

3.3.4.3 – Assessment of intestinal pathology

The response of the intestinal architecture to primary infection with *A. ceylanicum* in hamsters was much as expected and conformed to the pattern described earlier in this chapter (Fig. 3.10). Approximately similar lengths depths and crypts were recorded in all control-uninfected groups, which were killed on days 28, 49 and 63. As can be seen from Figure 3.30 villous height declined steadily from around 900 µm in the control-uninfected groups to as low as 13.5 ± 4.9 S.E.M. on day 63 after infection. Similarly, crypt depth increased with time from a depth ranging between 155.5 µm on days 49 and 63 and 169.24 µm on day 28 in control animals to as high as 860 µm on day 63 in the

Table 3.7. Experiment 7. (Recovery). The experimental design to investigate the effect of removal of *A. ceylanicum* worms from the intestine of hamsters on the cellular and architecture of the intestine.

No. of hamsters	Dose of L3 given	Mean number of worms recovered ± SEM	Day killed
5	Nil	Nil	28
5	Nil	Nil	49
5	Nil	Nil	63
5	50 L3*	0	35
5	50 L3*	0	42
5	50 L3*	0	49
5	50 L3*	0	63
5	50 L3	10 ± 2.145	28
5	50 L3	19.4 ± 4.545	35
5	50L3	14.8 ± 4.104	42
5	50 L3	9.2 ± 1.530	49
5	50 L3	8.4 ± 1.030	63

*Anthelmintic treatment (Ivermectin) was given to these groups on day 28 after infection and animals in each group were killed on days 35, 42, 49 and 63. The effect of ivermectin treatment was assessed measuring the egg output.

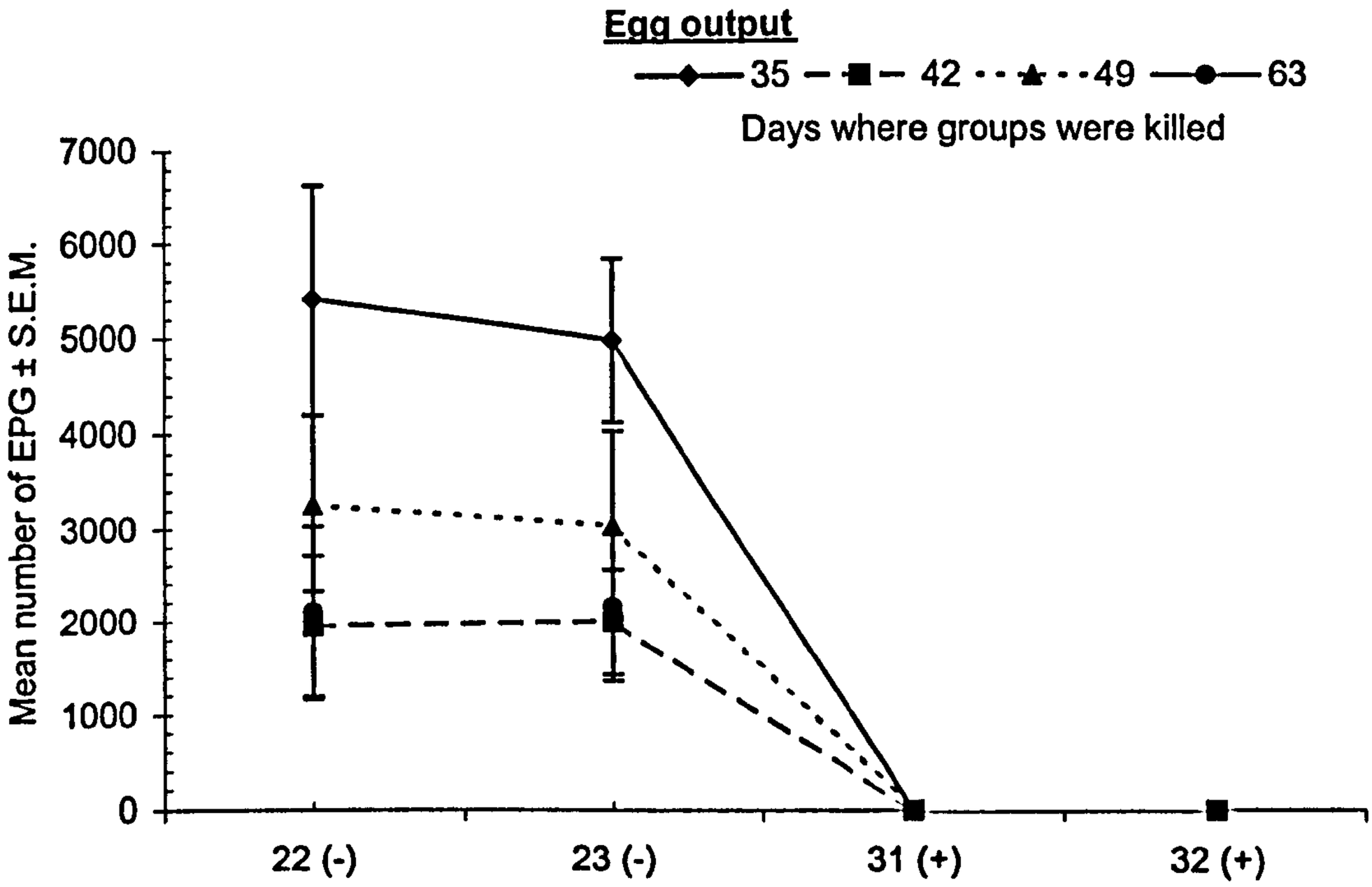


Figure 3.28 – Egg output from groups of hamsters treated with the anthelmintic drug Ivermectin. 22 (-) and 23 (-) are the days after infection and prior the administration of Ivermectin. 31 (+) and 32 (+) are the days after infection following treatment with Ivermectin.

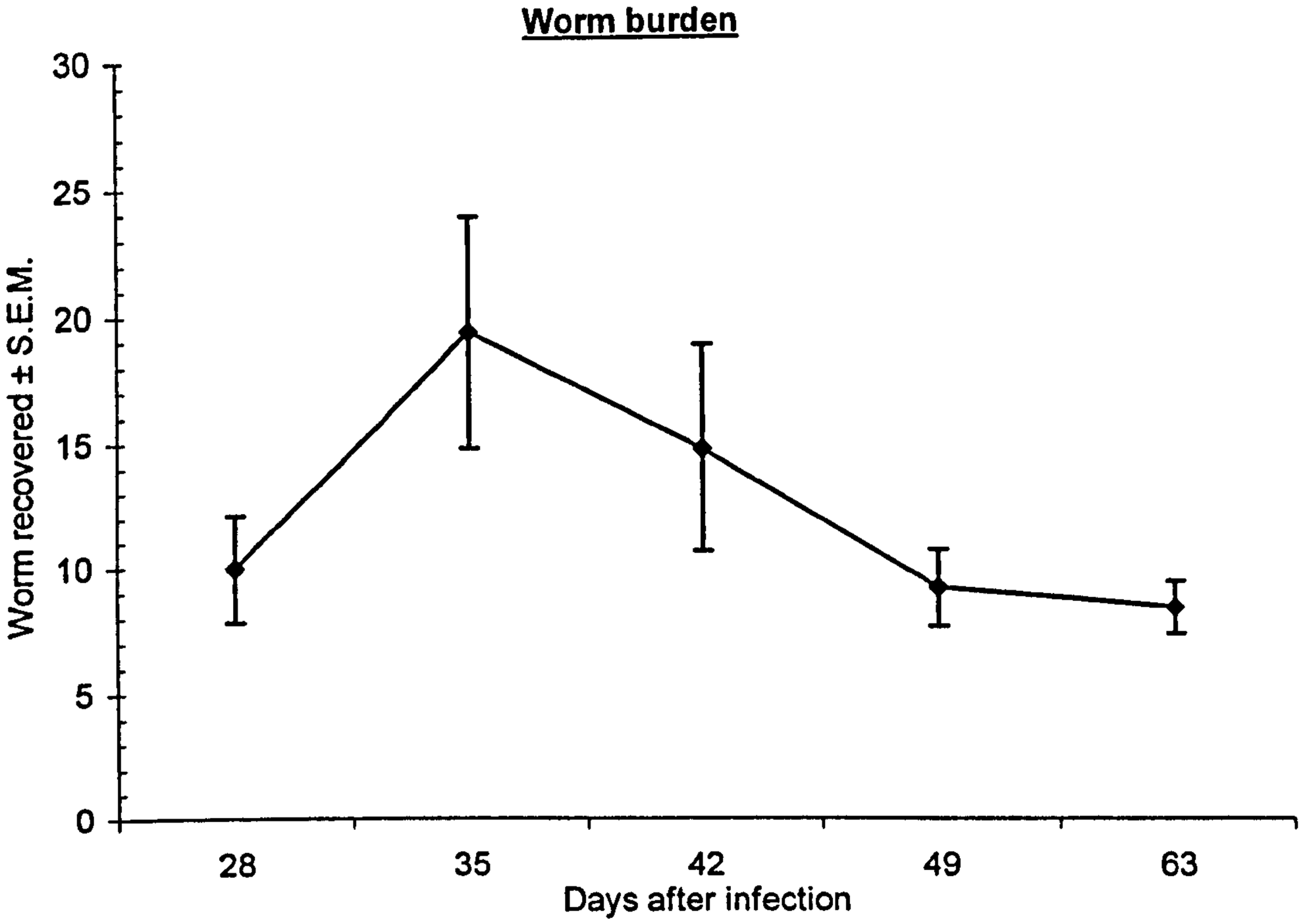


Figure 3.29 – Experiment 7. Recovery of adult worms from hamsters given primary infection of 50L3 *A. ceylanicum* on days 28 to 63.

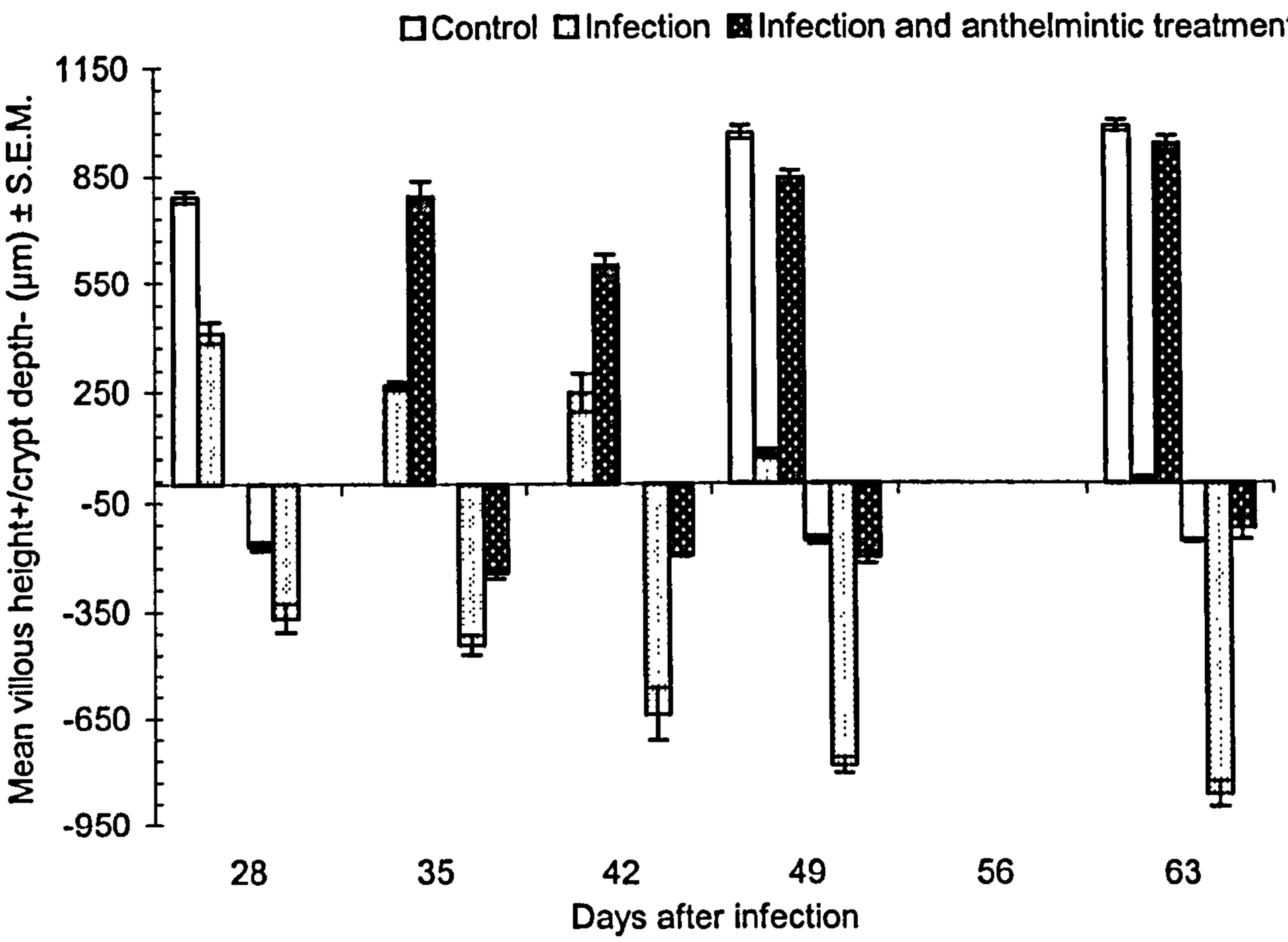


Figure 3.30 – Experiment 7. (Recovery). Changes in villous height (+) and crypt depth (-) in hamsters following infection and subsequent treatment with Anthelmintic.

Statistical analysis

Data were analysed by 2-way ANOVA with time (5 levels, days 28, 35, 42, 49 and 63) and treatment (3 levels, naïve, Primary infection and recovery) revealed:

Main effect of time on villi	($F_{4,48}$=6.759, P<0.001)
Main effect of treatment on villi	($F_{2,48}$=838.291, P<0.001)
Interaction between treatment and time	($F_{5,48}$=48.017, P<0.001)

Model R^2=0.972

Main effect of time on crypt	($F_{4,48}$=15.365, P<0.001)
Main effect of treatment on crypt	($F_{2,48}$=317.718, P<0.001)
Interaction between treatment and time	($F_{5,48}$=27.770, P<0.001)

Model R^2=0.931

infected animals. However, after removal of worms by Ivermectin, villous height and crypt depth began to recover within a few days and returned to a normal length comparable to that of the control animals by the end of this experiment. The effect of time on villous height and crypt depth was highly significant ($F_{4,48}$=6.759, P<0.001 and $F_{4,48}$=15.365, P<0.001 respectively) and similarly the effect of treatment on villous height and crypt depth were also significant ($F_{2,48}$=838.291, P<0.001 and $F_{2,48}$=317.718, P<0.001, respectively). Moreover, interestingly there were highly significant interactions between time and treatment on both villous height and crypt depth ($F_{5,48}$=48.017, P<0.001 and $F_{5,48}$=27.770, P<0.001).

3.3.4.4 – Cellular division in the crypt of Lieberkuhn

Changes in mitotic figures following primary infection and treatment with Ivermectin are shown in Figure 3.31. Overall, the number of mitotic figure observed in infected hamsters was higher than that found in naïve, control groups reaching the highest value on day 42 (24.1 ± 2.1 S.E.M.) then declining to reach 19 division per crypt ± 0.4 S.E.M. on day 63. In contrast, hamsters given Ivermectin, while still showing elevated numbers of mitotic figures a week after anthelmintic treatment began to show a return to levels comparable to those in naïve control animals after treatment in the following weeks. The return to baseline values was protracted and not quite completed on day 63, 5 weeks after treatment. The effect of time and treatment on cell division was highly significant ($F_{4.48}$=13.360, P<0.001 and $F_{2.48}$=121.054, P<0.005). In addition, the interaction between time and treatment was also significant ($F_{5.48}$=9.405, P<0.001).

3.3.4.5 – Mast cell responses

The number of MMC was assessed in this experiment from day 28 until day 63. The mean background count in naïve animals was 4 to 12 cells/mm^2 of tissue and in relation to this value all the primary infected groups had elevated

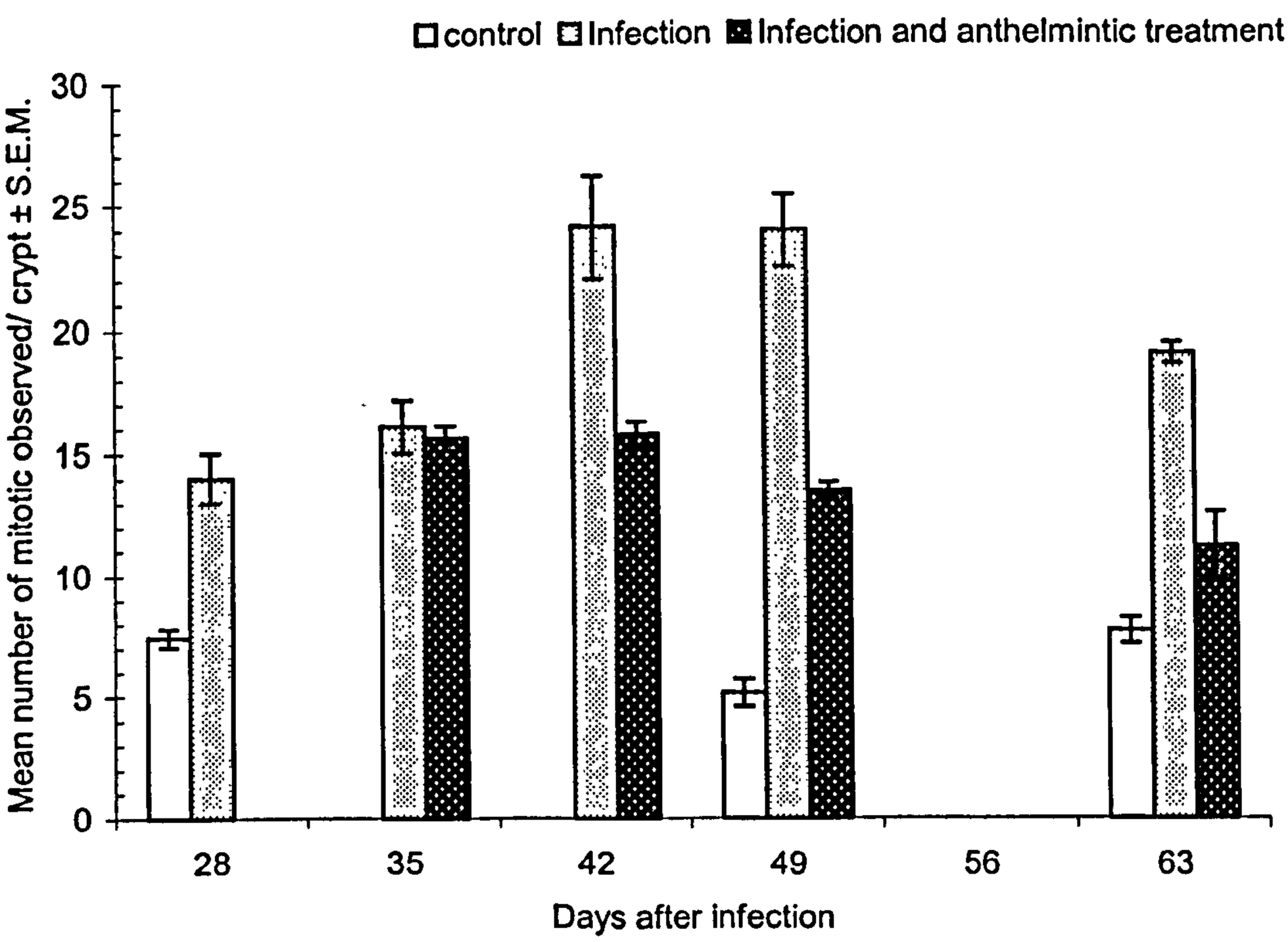


Figure 3.31 – Experiment 7. Changes in the number of mitotic figures in the crypt of Lieberkuhn in hamsters following infection and subsequent treatment with anthelmintic.

Statistical analysis

Data were analysed by 2-way ANOVA with time (5 levels, days 28, 35, 42, 49 and 63) and treatment (3 levels, naïve, Primary infection and recovery) revealed:

Main effect of time on mitotic	($F_{4,48}$=13.360, P<0.001)
Main effect of treatment on mitotic	($F_{2,48}$=121.054, P<0.001)
Interaction between treatment and time	($F_{5,48}$=9.405, P<0.001)

Model R^2=0.869

mast cells counts regardless of time of sampling. On the other hand, Ivermectin treated hamsters had 35.8 cells ± 2.8 S.E.M. on day 35 and 36.1 cells ± 1.8 S.E.M. on day 42 with a sharp reduction on days 49 and 63 returning almost to the value seen in naïve hamsters. These levels were lower than those found in the primary infected animals (Fig. 3.32). The time and treatment effects on mast cells were highly significant ($F_{4,48}$=43.728, P<0.001 and $F_{2,48}$=192.693, P<0.001 respectively). An interaction between time and treatment on mast cells was also highly significant ($F_{5,48}$=23.451 P<0.001).

3.3.4.6 – Goblet cell responses

Figure 3.33 illustrate goblet cell counts were again assessed in Experiment 7 weekly from day 28 to 63. Counts for all primary infected hamsters were elevated in relation to those for naïve animals (approx. 48 – 58). However, the number of goblet cells was as low as 69 cells/mm^2 ± 5.0 S.E.M. on day 28, 64.5 cells/mm^2 ± 3.6 S.E.M. on day 49 and 66.3 cells/mm2 ± 1 S.E.M. on day 63 which correspond well with values in naïve animals. Goblet cell counts then stayed relatively constant during the whole of this experiment after the worms had been removed by Ivermectin on day 28. The 2-way ANOVA showed a highly significant effect of time and treatment on number of goblet cells ($F_{4,47}$=12.565, P<0.001 and $F_{2,47}$=336.425, P<0.001, respectively). Furthermore, the interaction between time and treatment were also significant ($F_{5,47}$=4.962, P<0.01).

3.3.4.7 – Paneth cell responses

Normal uninfected hamsters showed higher numbers of Paneth cells and they were relatively stable over the course of the experiment (Figure 3.34). The number of Paneth cells in hamsters given the primary infection declined from 1.2 cells/crypt ± 0.2 S.E.M. on day 28 at the lowest 0.2 cells/crypt ± 0.1 S.E.M. on day 35, followed by an increase to reach 2.4 cells ± 0.1 S.E.M. on day 63. It was found that the number of Paneth cells declined when there were high

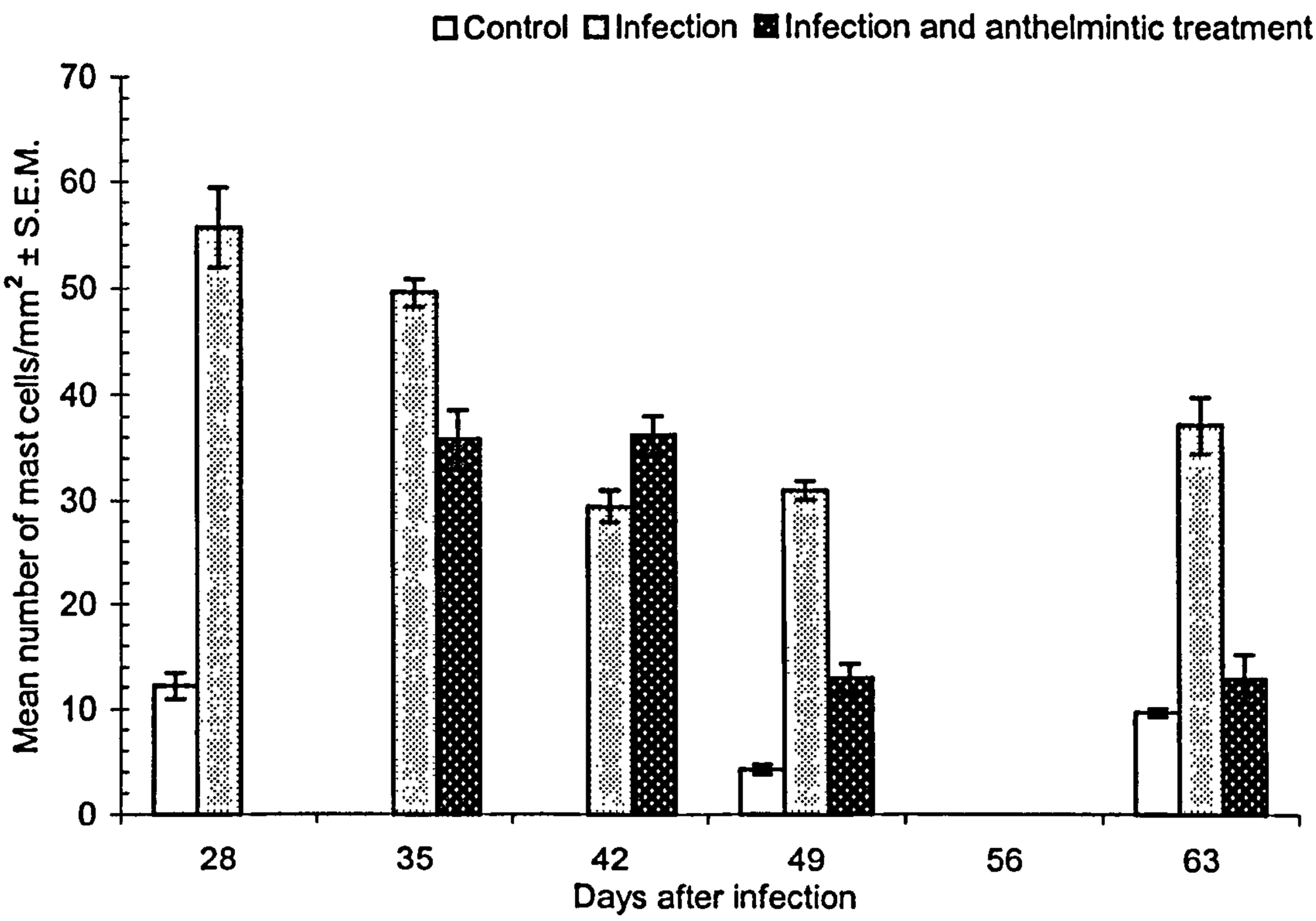


Figure 3.32 – Experiment 7. Mast cell response of hamsters to infection with *A. ceylanicum* and recovery after removal of worms by treatment with an anthelmintic drug.

Statistical analysis

Data were analysed by 2-way ANOVA with time (5 levels, days 28, 35, 42, 49 and 63) and treatment (3 levels, naïve, Primary infection and recovery) revealed:

Main effect of time on mitotic	($F_{4,48}$=43.728, P<0.001)
Main effect of treatment on mitotic	($F_{2,48}$=192.693, P<0.001)
Interaction between treatment and time	($F_{5,48}$=23.451, P<0.001)

Model R^2=0.931

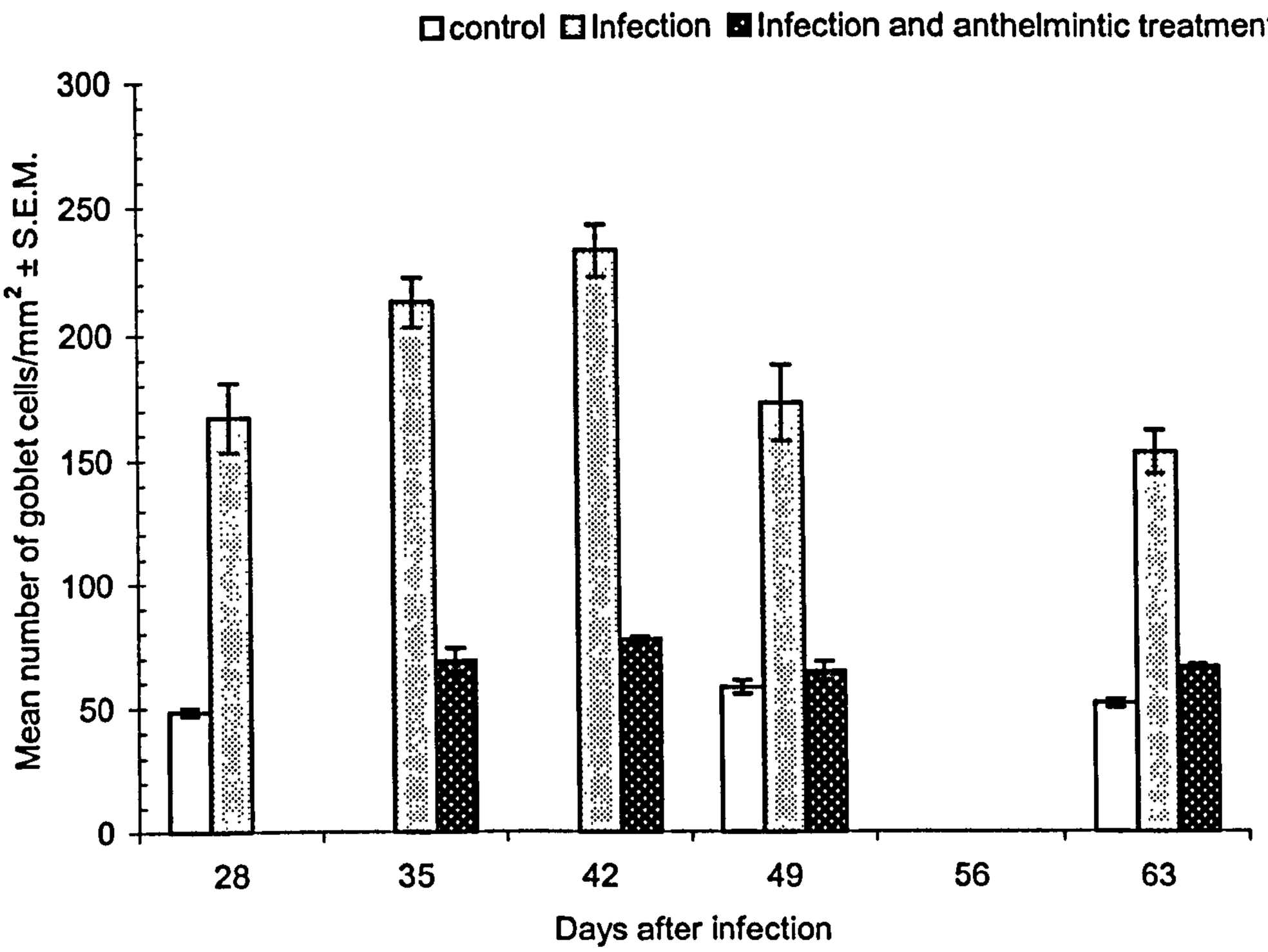


Figure 3.33 – Experiment 7. Goblet cell response of hamsters to infection with *A. ceylanicum* and recovery after removal of worms by treatment with an anthelmintic drug.

Statistical analysis

Data were analysed by 2-way ANOVA with time (5 levels, days 28, 35, 42, 49 and 63) and treatment (3 levels, naïve, Primary infection and recovery) revealed:

Main effect of time on mitotic	($F_{4,48}$=12.656, P<0.001)
Main effect of treatment on mitotic	($F_{2,48}$=336.425, P<0.001)
Interaction between treatment and time	($F_{5,48}$=4.962, P<0.001)

Model R^2=0.935

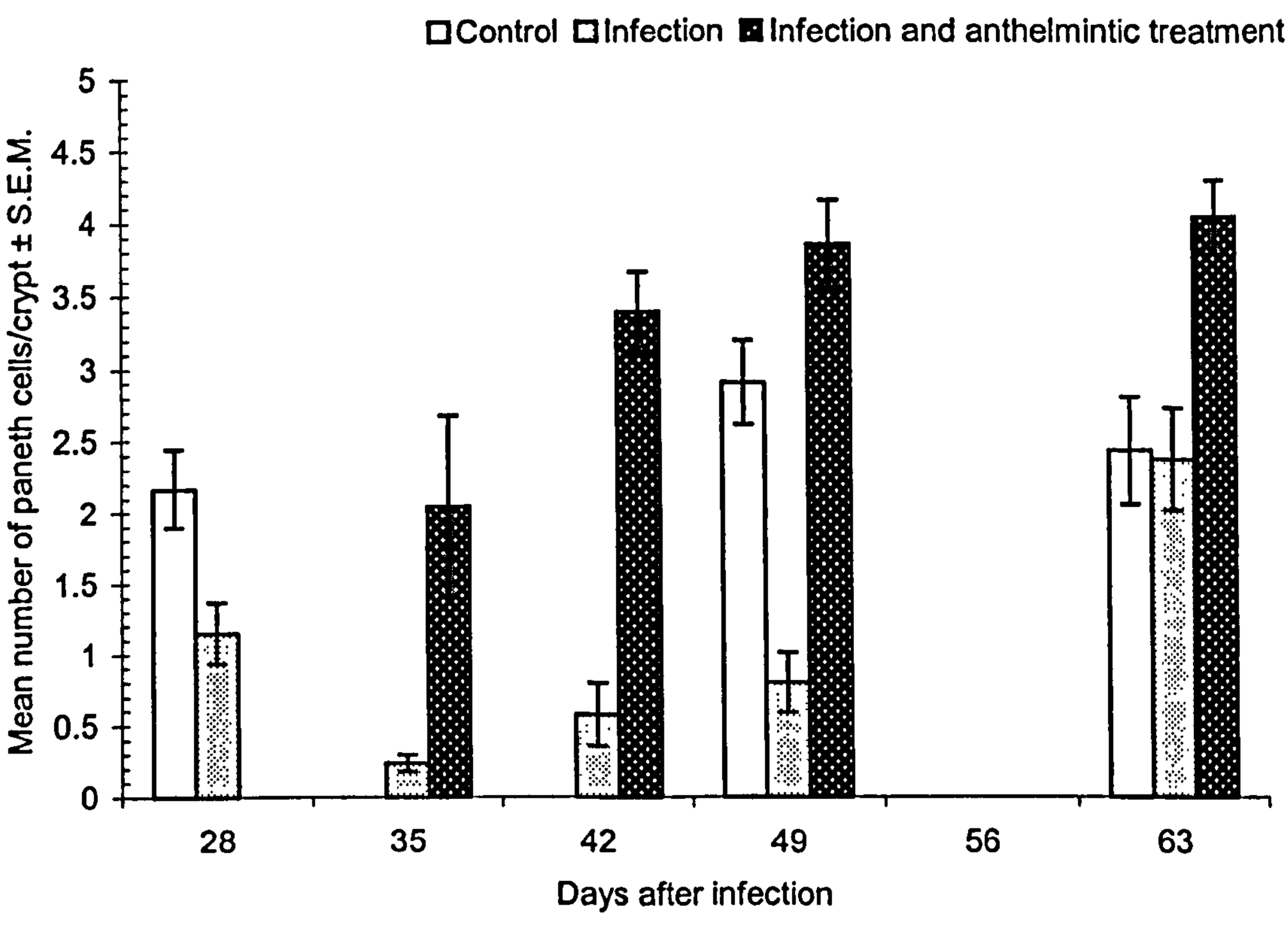


Figure 3.34 – Experiment 7. Paneth cell response of hamsters to infection with *A. ceylanicum* and recovery after removal of worms by treatment with an anthelmintic drug.

Statistical analysis

Data were analysed by 2-way ANOVA with time (5 levels, days 28, 35, 42, 49 and 63) and treatment (3 levels, naïve, Primary infection and recovery) revealed:

Main effect of time on mitotic	($F_{4,47}$=11.652, P<0.001)
Main effect of treatment on mitotic	($F_{2,47}$=56.405, P<0.001)
Interaction between treatment and time	($F_{5,47}$=1.557, P=0.191)

Model R^2=0.737

numbers of worms in the intestine. However, the more interesting finding is that the number of Paneth cells rose up to exceed the number found in naïve hamsters after animals were treated with anthelmintic (Figure 3.35). Paneth cell numbers were significantly affected by treatment ($F_{2,47}$=56.405, P<0.005) and by time ($F_{4,47}$=11.652, P<0.005). However, the interaction between time and treatment was not significant ($F_{5,47}$=1.557, P=0.191).

3.4 – DISCUSSION

The experiments reported in this chapter have clearly shown that marked changes occur in the intestines of hamsters infected with *Ancylostoma ceylanicum*. Changes were detected at the levels of cellular proliferation, infiltration of the mucosa by inflammatory cells such as mast cells and eosinophils, up regulation of goblet cells and in the intestinal architecture. They were dependant on both the presence of worms, the intensity and the duration of the infection. Up regulation of goblet cell and mast cell numbers have been reported previously (Garside, 1989), but changes in Paneth cell and eosinophil numbers in the intestinal mucosa of hookworm-infected animals are reported here for the first time. Likewise, there are no earlier experiment based reports on quantitative data on architectural changes in the intestinal mucosa during hookworm infections.

It has been reported that local infiltration of the mucosa by inflammatory cells generates a hostile environment in the intestinal gut and that this process is regulated by cytokines which are released by lymphocytes after activation by antigens liberated from nematodes (Artis *et al.*, 1999; Butterworth, 1984). It is generally accepted that CD4+ T cell play a role in immunity to gastrointestinal nematodes, with the T helper 2 (Th2) subsets mediating the host protective response (Betts *et al.*, 2000; Doolan and Hoffman, 2000; Pritchard, 1995b), although evidence from human infections is not yet conclusive. The infection in canine hosts with related hookworms provide good evidence that parasites are controlled by the host immune responses (Miller, 1971). The effector mechanism is still not understood but the inflammatory phase is known to act non-specifically since unrelated parasites, concurrently resident in the intestine

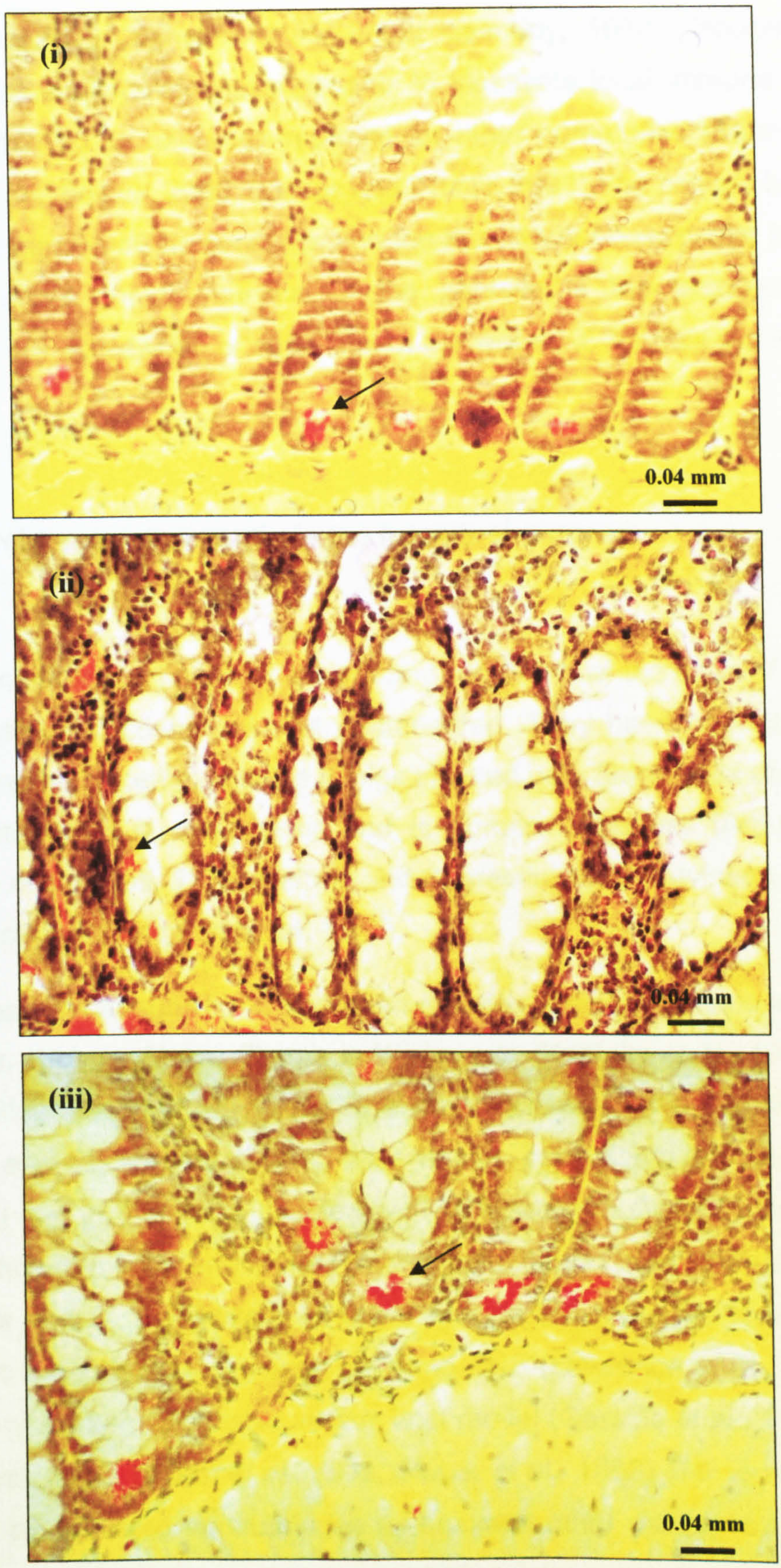


Figure 3.35 – Sections from intestinal tissue of hamsters show the Paneth cells (pointed in black arrows) in the crypt of naïve uninfected animals on day **(i)** and during primary infection with *A. ceylanicum* on day 35 **(ii)** and in the infected and treated hamsters with Ivermectin on day 49 **(iii)**. (Magnification 200 X).

are also rejected (Behnke *et al.*, 1992; Kennedy, 1980). Hookworms may secrete immunomodulatory components to inactivate local immune responses (Behnke, 1987a) as has been reported for other nematodes (Behnke, 1987b; Hartmann and Lucius, 2003; Loukas and Prociv, 2001) The contest between the evasion strategies of the parasites and the local immune response by the host, result in either chronic survival of the parasite, if the immunomodulatory strategies are successful, or expulsion of the worms if the host gains the upper hand.

3.5.1 – Architectural changes in the intestinal mucosa

Changes in the height of the villi and the depth of the crypt of Lieberkuhn during enteric infections are well documented. They have been described in different model systems, such as in pigs infected with *Salmonella cholerae*-suis, rats infected with *N. brasiliensis* and in human Giardiasis (Arbuckle, 1975; Ferguson and Jarrett, 1975; Geffroy and Segrestin, 1972; Levinson and Nastro, 1978; MacDonald and Ferguson, 1977).

Intestinal changes have also been reported during infections with parasitic helminths, such as *N. brasiliensis* in Wistar rats (Koninkx *et al.*, 1988), *Ascaris* in pigs (Stephenson *et al.*, 1980), *Echinostoma revolutum* and in BALB/c mice (Bindseil and Christensen, 1984), mice with *A. ceylanicum* infection (Carroll *et al.*, 1986) and in C57 mice infected with *Hymenolepis diminuta* (McKay *et al.*, 1990) although most research has been conducted on intestinal worms such as *T. spiralis* and *N. brasiliensis*. In these models the reduction in villous height and crypt hyperplasia have been linked to TNF (Lawrence *et al.*, 1998), as in the case of studies using graft versus host model (Garside *et al.*, 1993; Garside and Mowat, 1993; Stuber *et al.*, 1999; Stuber *et al.*, 1999) TNF is believed to be released by activated macrophages and several other cell types as part of the Th2 dependent response to the presence of worms.

Experiments with *T. spiralis* have shown that worm expulsion can occur in the absence of changes in villous height and crypt depth, and hence in this

species expulsion of worms is not dependant on the TNF driven process that induce architectural changes to the gut mucosa. In the case of hookworm infections, there is a complicating factor, namely that the worms feed on the mucosal tissues and hence loss of villi may be partly due to worms feeding as well as to any accompanying immune response (Lawrence *et al.*, 1998).

In the experiments reported in this chapter, the usual range in villous height of uninfected, naïve hamsters was between 900-1200 µm (Experiment 1. Figure 3.5 (A), Experiment 3 and 4. Figure 3.10 (A and B) and Experiment 5. Figure 3.21 (A). In Experiment 3 and 7, the lower values in villous height suggested that, despite all of the precautions taken, they were experiencing some other enteric infection. However, by week 2 and 3 in both experiments respectively, villous height had been restored to its normal range. Excepting the low values on day 0 in Experiment 3 and possibly Experiment 5 and day 28 in Experiment 7, the values in control naïve hamsters throughout the remainder of the experiments showed little variation, thereby establishing a reliable base line against which to assess changes associated with hookworm infections.

It is very clear from the results in Fig (3.10. A and B) that villous height declined within 2 weeks of infection. There was little evidence of a decline any earlier, even when worm burdens were as high as 118 worms (Experiment 5, day 12) and hence they become detectable only when the worms moult to the pre-adult stage. This moult is known to occur on days 7-11 (Garside and Behnke, 1989; Ray *et al.*, 1972a). Since there is little evidence of anaemia earlier in the infection (Garside and Behnke, 1989; Menon and Bhopale, 1985), deep grazing on the mucosa with associated blood loss is probably only associated with the pre-adult and adult stages of *A. ceylanicum*. Therefore, the slow decline of villous height is consistent with the onset of grazing on the mucosal surface by adult worms.

Interestingly, adult worms were completely removed from the intestine of the hamsters following the oral administration of Ivermectin, which is an anthelmintic drug that is often used to reduce or eliminate parasitic infections (Behnke *et al.*, 1993; Klei *et al.*, 2001; Monahan *et al.*, 1996; Monahan *et al.*, 1997). This resulted in a quick recovery of the villous height from the height of

267 µm ± 11.1 S.E.M. on day 35 of infection to a length of 790 µm ± 43.8 S.E.M. and this increased further to reach near the approximate height of the normal villi, which was around 935 µm, by day 63. This recovery in villous height together with crypt depth is illustrated in Experiment 7 (Figure 3.30).

The experiments revealed a concurrent increase in the depth of the crypts and in mitotic activity in the enterocytes of the mucosa and both of these are unlikely to be a direct consequence of worm feeding. Rather, as in other intestinal infections and together with the reduction in villous height, these changes are more likely to reflect a Th2 mediated mucosal response to infection with worms. As was demonstrated in Figure 3.12 (A and B) crypt depth increased steadily with the duration of infection and was partly dose dependant (Figures 3.22 of Experiments 5 and 6), but there was also evidence of a ceiling effect. Kamal (2001) suggested that the crypt hyperplasia may be a consequence of both goblet and Paneth cell hyperplasia. Moreover, hyperplasia by these cells, as they located exuberantly in the crypt, may reflects a general increase in stem cell division. It has been shown that crypt hyperplasia occurs when there is an increase in the number of mitotic cells per crypt (Lawrence *et al.*, 1998) and so presumably does goblet and Paneth cell hyperplasia.

The results of Experiment 7 in figure 3.30 showed that when worms were removed using Ivermectin, the depth of the crypt returned to normal within a few days following treatment and it was soon similar to that in control uninfected groups. Therefore, the results from the last experiment suggest that changes in crypt depth are more likely to reflect Th2 dependant processes. Reference to Figure 3.22 shows that despite a wide range of worm burdens on day 30 pi. Crypt depth ranged from 500-600 µm. In fact, very comparable values were also evident on day 20 particularly if more than 5 worms were present, and this is largely in agreement with the time course data Figure 3.10. In comparison, experiments with *T spiralis* in mice showed that the crypt response peaked on day 14 and that it declined by days 27. This peak was at a time when most worms had been lost from the intestine (Kamal, 2001).

In the experiments described here worm burdens were maintained for considerably longer than *T. spiralis*, and this is consistent with the known

chronic survival of *A. ceylanicum* in hamsters (Garside and Behnke, 1989). It would appear likely therefore that unlike in *T. spiralis*, in the case of the hamsters infected with *A. ceylanicum*, the mucosal response is sustained for a very long time, in fact as long as the worms reside in the gut. Examination of the worm burden data from Experiment 3 suggests that there was a gradual slow loss of worms over the course of 6 weeks with a reduction of 61% between days 7 and 42 pi. Nevertheless, villous height continued to decline and crypt depth to increase throughout. If the changes were solely the consequence of feeding by the worms, we might have expected some recovery as worm burden fell, particularly towards weeks 6 and 7. One explanations of this is that the host was gradually gaining the upper hand and with loss of part of the worm burden, the intestinal response was less inhibited and could increase in the intensity. Also, dose related changes in villous height argue against the idea that villous height is primarily attributable to worm feeding (Fig. 3.21). On day 20 for example, when the worms are adult and the infection is several days into patency, despite an overall negative relationship between worm burden and villous height, when the uninfected animals are excluded from consideration the relationship becomes very weak. In fact, in the range 5 to 35 worms, villous height is very similar. We might have expected 35 feeding worms to abrade the intestinal mucosa to a considerably greater degree than just 5. With the results from the last experiment, these observations suggest that changes in villous height and crypt depth are attributable to more than one factor.

3.5.2 – Mast cells

It is well known that mucosal mast cells play a very important role in immunity against parasitic infection which is believed to be induced by antigen-activated T cells (Roitt *et al.*, 1998). During infection with parasitic nematodes, there is generally a marked intestinal mastocytosis as reported by Alizadah and Wakelin (1982a), Dehlawi (1986) and others (Else and Finkelman, 1998; Grencis *et al.*, 1993; McDermott *et al.*, 2003). Mastocytosis was also observed here during this work with hamsters infected with the hookworm *A. ceylanicum*.

Mastocytosis has been reported during many gastrointestinal parasitic infections (Carroll *et al.*, 1986; Dehlawi, 1986; Featherston *et al.*, 1992; Garside *et al.*, 1990; Marshall and Bienenstock, 1994). The increase in mast cells is associated with the process of worm expulsion from the intestine in some species but not all. Some reports showed that mastocytosis can also be induced in rats infected with *N. brasiliensis* and in mice infected with *T. muris* in the absence of worm expulsion (Jarrett *et al.*, 1968; Lee and Wakelin, 1982). In fact mastocytosis is known to be a useful element in the expulsion of *T. spiralis* and S. ratti from rodents (Gause *et al.*, 2003; Ha *et al.*, 1983; Nawa and Korenaga, 1983) but not in the case of *N. brasiliensis* (Mitchell and Wescott, 1983). Mast cell deficient animals fail to resist *T. spiralis* and *S. ratti* but can expel *N. brasiliensis* (Ha *et al.*, 1983; Mitchell and Wescott, 1983; Uber *et al.*, 1980). The possible effector molecules in mast cell-mediated adult worm expulsion came from goblet cells in hamsters. Unlike in mice and rats, the expulsion of S. venezuelensis in Syrian golden hamsters is associated with massive goblet cell hyperplasia and the production of large quantities of mucins (Ishikawa *et al.*, 1995). The mucin of goblet cells in four species of hamsters shown to be highly sulphated but the degree of sulphation determined the rapidity of expulsion (Ishikawa *et al.*, 1995). As the mast cell granules contain a highly sulphated molecular weight of proteoglycans, it was tempting to propose that this sulphated proteoglycans play the effector molecule which prevents the establishment of the nematode, such as adult *Strongyloides*, and thus mediate their expulsion (Onah and Nawa, 2000). Furthermore, it was found by Maruyama *et al.*, (2000) that mast cells contribute to the expulsion of *S. venezuelensis* from mice by preventing the invasion of the host intestinal mucosa by the adults since treatment with glycosaminoglycans produced by mast cells, such as heparin, dextran sulphate and ChS-E, inhibited the binding of *S. venezuelensis* adhesion molecules to mucosal epithelium and thus the invasion of the gut mucosa by adult in *in vivo*. Previous findings could also be addressed in our results. Nevertheless, in situations where mast cells appear to play an important role in worm expulsion, the precise mechanism of the mediation was less clear (Onah and Nawa, 2000)

Work presented in this chapter mainly confirmed the idea that mast cells were elevated throughout primary infection with *A. ceylanicum* and responded to the presence of the nematode in the intestine. The activation of mast cells which are then capable of initiating inflammatory events through the release of performed mediators such as histamine, serotonin, proteinases and some other inflammatory mediators such as leukotrienes and prostaglandins are believed to be specific T-dependant processes (Befus and Bienenstock, 1979; Grencis *et al.*, 1993; Lawrence *et al.*, 1998).

In the experiments described in this chapter, the numbers of mast cells in uninfected, naïve hamsters were generally about 4-16 cells/mm^2 of intestinal tissue (Figure 3.14. B and Figure 3.32). Most experiment showed some variations between the mean number of mast cells in the uninfected, naive animals which may be attributed to some host variation or some enteric pathogens that stimulate mast cells to proliferate in the tissue. A dramatic mast cell hyperplasia was evident in the intestine of hamsters a few days after infection. However, the number of mast cells in naïve hamsters remained within a range of variation that was distinct from that found in infected hamsters, thereby establishing a base line against which to assess changes associated with the presence of hookworms in the intestine.

It can be seen from the results in (Figures 3.6 and 3.14 A and B) that mastocytosis occurred in the hamster intestines infected with *A. ceylanicum* within several days of infection. The onset of mastocytosis was just a few days after infection to reach the peak of 47.461 cells/mm^2 on around day 21-28 after infection (Figure 3.1.4. B). Although the worm burdens recovered from Experiments 3 and 4 were different and with more variations in Experiment 4, the increase in mast cells number was very similar.

3.5.3 – Goblet cells

As with mastocytosis, goblet cell responses during hookworm infection were dramatic. Goblet cells are mucosal cells that secrete mucus, which

comprises a family of a high-molecular-weight glycoproteins collectively known as mucins (Forstner, 1978). The mucus plays several important roles in the intestinal mucosa. Finally, it helps to protect the mucosal lining by acting as a lubricant and hence mucus is continuously secreted by goblet cells in the mucosa. Even uninfected animals show competitive secretion of mucus. Moreover, this can be up regulated by elements of the immune response. Thus Th2 lymphocytes secrete IL-13 which helps to provoke and up regulate goblet cells in the mucosa. Recent studies have also suggested that goblet cells differ from mast cells which known to be bone marrow derived. They arise by mitosis from multipotential stem cells at the base of the crypts (Cheng and Leblond, 1974c) and are more likely to be controlled by local factors including T-cells (Miller, 1984). Moreover, more recent studies by Kamal (2001) using Cyclosporin A in *T. spiralis*-infected mice showed inhibition in goblet cells which again confirmed that goblet cells are a T cell driven component.

Most experiments in this chapter revealed little differences in goblet cell numbers between uninfected controls and infected hamsters within the first 7 days of infection. Similar concentration of goblet cells in control and infected hamsters in the first week of infection were also seen by Garside (1989) who stated that there was no significant increased in goblet cells on day 7 pi. This suggests that goblet cell hyperplasia is generated by the pre-adult and adult stage of A. ceylanicum rather than L3 and L4 larvae.

Goblet cell hyperplasia is a feature of many GI nematode infections and the protective function is considered to be mediated through its associated increased mucus production which exclude and trap worms (Levy and Frondoza, 1983; Miller and Nawa, 1979b; Onah and Nawa, 2000) and prevents their establishment by interrupting contact with the gut mucosa (Miller, 1987) which eventually expel the nematode from intestine. It was found that the qualitative changes in goblet cell mucins, including the terminal sugar of the mucins produced and secreted by the goblet cells, are more important in the final expulsion of the adult *N. brasiliensis* from the intestine of their host than the quantitative changes (Ishikawa *et al.*, 1993; Oinuma *et al.*, 1995). The evidence which shows that goblet hyperplasia occurs in rodents with *T. spiralis* or *N. brasiliensis* infection at the time of worm expulsion (Garside *et al.*, 1992;

Ishikawa *et al.*, 1994; Nawa *et al.*, 1994) may also be relevant here during these experiments. It was found that the number of goblet cells increased in the hamster intestines when *A. ceylanicum* begun to be lost. However, in some cases, such as in Experiments 3 and 4, there was no direct relationship between the decline in worm burden and goblet cell hyperplasia.

The final experiment reported in this chapter indicated that following the removal of worms by treatment with Ivermectin, goblet cell numbers dropped rapidly to levels typical of naïve control animals. In fact, goblet cell numbers in infected-treated animals were almost back to control levels within 7 days. This suggests that the maintenance of goblet cell hyperplasia is highly dependant on continuous signaling through regulatory pathways. When worms are lost, as for example after treatment with Ivermectin, the absence of worm antigens and local stimulation of the mucosa, must result in cessation of the afferent signals that provoke local T cells to secrete cytokines such as IL-13. The observation that goblet cell numbers decline so rapidly also suggests that individual goblet cell do not live long, and in this case once they are lost, or have secreted their products, they are not replaced in numbers comparable to those see at the height of infection in infected animals.

The last experiment revealed that a quick and complete recovery followed removal of the worm by Ivermectin. Figure 3.33 showed that the numbers of goblet cell in all infected groups of animals were approximately at the same level as in naïve-uninfected groups. Therefore, it can be suggested that the up regulation of goblet cells is mainly through factors presented by adult worms in the intestine.

3.5.4 – Paneth cells

Paneth cells are believed to play an important role in intestinal immunity against different pathogens as for example in bacterial infection (Ayabe *et al.*, 2000; Lopez_Boado *et al.*, 2000; Rodning *et al.*, 1982; Rodning *et al.*, 1976). They are also thought to be involved in digestion through a Trypsin-like product

which is released from human Paneth cells that digest food elements (Bohe *et al.*, 1984) and in the proliferation and differentiation of intestinal epithelium (Poulsen *et al.*, 1986). Their numbers are known to change during intestinal infections but the precise role during helminth infection have not been elucidated (Kamal, 2001) and yet need to be understood.

Sections of the small intestine from DSN hamsters stained with phloxin-tartrazine showed differences in Paneth cells numbers and types. Subbuswamy (1973) reported that yellow granules reflected immature Paneth cells whereas mature cells were characterised by red-coloured granules when stained with Phloxine-tartrazine. The two types of Paneth cells were also reported by Kamal (2001) using expression (Tartrazine positive, Phloxine negative) in case of yellow Paneth cells and (Tartrazine negative, Phloxine positive) of the red Paneth cells. Moreover, using this distinction, Kamal (2001) found that immature Paneth cells were the dominant cells in the intestine of uninfected mice. However, it was also found that these cells became more obvious by day 8 during infection in which they reached their maturity. Cells remain in the intestine and carry out their functions by the release of their contents into the lumen until renewal, which occurs every two to three weeks (Cheng and Leblond, 1974a). However, in the case of hamster experiments presented in this thesis, both types of Paneth cells were recognised but not distinguished and Paneth cell counts therefore reflect both stages of developments.

It has been reported that alterations in Paneth cell numbers in the intestinal tract are associated with some parasitic infections (Kamal, 2001; Lewin, 1969a; Lewin, 1969c). The Paneth cell is a distinctive cell and has never been shown to develop from the transformation of the other glandular cells of the mucosa. It was suggested by Lewin (1969c) that these cells are most likely to originate in the normal and diseased colon from stem cells *in situ*. Data from work by Miyake, *et al.* (1995) suggested that the regeneration of Paneth cells following elimination using dithizone treatment seems to occur without cell division taking place and may be due to a direct transformation of crypt columnar epithelial cells or a high rate of differentiation of crypt stem cells into Paneth cells. However, crypt stem cell kinetics and the role of Paneth cells in this context are still not been elucidated.

The results presented in this chapter generally indicated a reduction in total numbers of Paneth cells following infections in hamsters. This may be explained by degranulation in the intestinal crypt of hamsters during infection such that mean values were lower than in uninfected controls. When the initial worm burden was high, as in Experiment 3, Paneth cell numbers declined as early as day 7 pi; and then as worm numbers declined over the subsequent 5 weeks, Paneth cell numbers returned to normal. When the initial worm burden was low (Experiment 4), there was no immediate evidence of a reduction in Paneth cell numbers but this declined as the infection progressed into the 4 and 5th weeks. There was also support for a dose-related reduction in Paneth cell numbers from experiments 5 and 6. On day 30 there was a highly significant negative relationship between Paneth cell counts and parasite burden (Figure 3.26 – B), although at earlier time this relationship was not as strong as that reported on day 30 (Figure 3.26 – A) and similarly, on day 20 of experiment 6 (Figure 3.2.7 – C). Therefore, it could be added that as changes in Paneth cell responses are time dependant, the level of response may also differ according to the number of worms present in the intestine with later stages of infections showing much more marked depression in Paneth cells. Moreover, there was no differences in the mean Paneth cell numbers between uninfected hamsters and those, which received 50 L3 of *A.ceylanicum* on day 12 (Figure 3.26-D). However, as seen in Fig 3.26 – B & C, there was a reduction in the numbers of Paneth cell was higher when establishment of worms in the intestine of hamsters. Therefore, it could be suggested that Paneth cells may also be partially dose dependant.

A reduction in the total number of Paneth cells has also been reported in other hosts with different infections. Creamer and Pink (1967) showed that there was a reduction in numbers of Paneth cells in patients with coeliac syndrome. Also, study by Rose, *et al.* (1992) showed that a reduction in Paneth cells in the intestine of immunised NIH mice with *E. vermiformis* infection. However, data from NIH and BALB/c mice infected with *T. spiralis* revealed completely contrasting results. During this infection, Paneth cell numbers peaked and was significantly increased 8 days after infection in most part of the intestine with the exceptional of the response in the ileum where the peak was at day 14 pi

compared to uninfected control mice. This increase was followed by a slow reduction towards the end of the experiment. As shown in Fig 3.8, the mice exposed to *T. spiralis* in these experiments also showed an increase in Paneth cell numbers. These differences in response between mice and hamsters are interesting. It could be due to differences in the experiment models employed by the various workers. However, another possibility is that host species differ in their responses. In fact, this is the reason why some hamsters were infected with *T. spiralis* in some of the current experiments. Although in general the *T. spiralis* infectivity was lower than had been intended, it is very clear from Experiment 2 that whilst Paneth cell numbers declined in hamsters subjected to both *T. spiralis* and *A. ceylanicum*, they increased by day 12 and 22 in mice. This suggests that there are fundamental differences in the hamster's and the murine responses. One explanation of there observations is that mice are able to up regulate Paneth cell numbers in response to infection. In contrast, in hamsters, Paneth cells may degranulate and not be replaced rapidly or alternatively, that newly formed Paneth cells in hamsters release their contents rapidly after maturation, giving the overall impression of a reduction. Thus low numbers of granule containing cells may not be matched by appearance of new granule containing cells. Data from Satoh, *et al.* (1990) showed that Paneth cells differ structurally between different species of animals and therefore, it is possible that Paneth cells of other species play a role another functions. It was also reported that Paneth cell secretions in rats and mice appear to be stimulated by changes in the bacterial milieu of the intestine (Satoh, 1988a; Satoh *et al.*, 1986) and it is possible that the bacterial population differ in hamsters.

In fact, there is evidence for this suggestion because when hookworms were removed by treatment with Ivermectin, Paneth cell numbers increased, not only returning to normal, but also exceeding normal values. This observation supports the idea that Paneth cell are being reduced in infected hamsters and that their generation is in fact increased during infection but that they cannot be detected by staining because they degranulate as soon as they are mature. It is also possible that treatment with Ivermectin reduced the bacterial activities in the intestine and therefore the values in treated animals exceeded the values in

normal uninfected groups. On loss of the signals for degranulation, the number of non-degranulated cells accumulated and exceeded those seen in control uninfected hamsters.

3.5.5 – Eosinophils

Eosinophilia is generally regarded as a hallmark of helminth infection and is well recognised in humans and animals infected with hookworms (Behm and Ovington, 2000; Garside *et al.*, 1989; Meeusen and Balic, 2000; White *et al.*, 1986). However, much less is known about the accumulation of eosinophils in the intestinal mucosa during infection.

The numbers of eosinophils in the intestine were only quantified in Experiments 6. Changes in the number of eosinophils in the intestinal mucosa were detected during primary infection of *A. ceylanicum* with a dramatic increase from a very low level to marked infiltration in infected animals. The data presented in figure 3.2.8 shows a trend of increasing eosinophil counts with increasing worm burden. Earlier, Grove (1974) reported increases in the eosinophils in the blood during hookworm infection. Eosinophilia, the presence of the elevated numbers of eosinophils in the circulation is generally considered to reflect the mobilisation of eosinophils from their places of storage in the bone marrow by the IL-5 from activated T cells. The mobilised cells then infiltrate tissue site where helminths reside. Although the mechanism by which eosinophil-mediated protection against helminth infection still not fully understood, it appears that this mechanism involves antibody-induced release, complement-induced release or both toxic granule proteins and reactive oxygen intermediates by activated eosinophils. Evidence suggests that there might be significant differences in the susceptibility to and mechanism of eosinophil-mediated killing between different life-cycle stages of the same parasite (McDermott *et al.*, 2003). Moreover, it was found that eosinophils can kill the larval stage of some helminths, as in case of *T. spiralis*, *in vitro* but the result from *in vivo* experiments remain controversial (Klion and Nutman, 2004). In the present work, the dose response experiment, whilst showing an increase in

mucosal eosinophils in infected compared with naïve-uninfected animals, did not indicate any clear trend that was dependant on the parasite burden. Thus, it may be sufficient for IL-5 to be triggered by the presence of worms and the response is then mobilised irrespective of the worm burden. Nevertheless, more investigation of the role of eosinophil needs to be carried out in this field.

In other helminth models, a relationship has been established between the eosinophils and infections (Ackerman and *al*, 1990; Butterworth and Thorne, 1993; Garside *et al.*, 1989; Grove *et al.*, 1977; Meeusen and Balic, 2000; Rainbird and *al*, 1993). It has been observed that the resistance of inbred strain of mice to infections with *T. muris* (Else and Grencis, 1991a) and *N. brasiliensis* is positively correlated with their capacity to generate tissue eosinophilia after infection. Similarly, it was reported that several independently selected lines of sheep, bred for increased resistance to gastrointestinal parasite infections, also showed greater eosinophil responses after infection compared with random-bred or low response flocks (Buddle *et al.*, 1992; Dawkins *et al.*, 1989; Gill, 1991). However, the correlations observed in these experiment were only after priming of sheep with natural or experimental infections. More likely, the presence of eosinophil activities in the gastric lymph of sheep infected with *O. circumcincta* was correlated with worm burden, but only 2-3 days after secondary, and not after primary, infections (Stevenson *et al.*, 1994)

Finally, the experiments reported in this chapter have established that following infection with *A. ceylanicum,* the intestinal mucosa of hamsters undergoes marked changes, reflected in both the mucosal architecture and in infiltration by inflammatory cells. These changes were dependant on duration and intensity of infection and on the presence of worms since removal of worm by chemotherapy resulted in a return to a baseline values for most of the parameters recorded. Differences in the rate at which changes occurred in specific component of inflammation suggest that the different elements may be controlled independently to some degree, although centrally driven by the Th2 response. Perhaps the most remarkable observation was that some adult worms survived to the end of each experiment, despite the intense, sustained inflammatory response in and around their sites of attachment.

CHAPTER FOUR

ACQUIRED IMMUNITY; CHALLENGE & SUPERIMPOSED INFECTION

4.1– SUMMARY

Two experiments are reported in which hamsters were exposed initially either to an abbreviated primary infection and then challenged with *A. ceylanicum* or to a continuous primary infection during the course of which the challenge infection was superimposed. Marked changes to the mucosal architecture and the associated cellular parameters were observed. In hamsters where the primary infection had been removed prior to challenge, continuous reduction in villous height was observed from day 10 post challenge, together with an increase in crypt depth relative to the normal range. In hamsters subjected to the superimposed challenge, villous height was already low on day 10 pi and there were some signs of a return towards control values, but even after 3 weeks the villi had not returned to baseline values and the crypts were still deeper than in controls. Hamsters given the challenge infection after termination of the immunising infection responded with a slow increase in mitotic figures in villi, whereas this increase was more marked initially in hamsters given the superimposed infection. Mast cell responses were moderate in the challenged hamsters but were high in the hamsters given superimposed infection, mainly because of persisting high mastocytosis from the original primary infection. A goblet cell response was also evident in the challenge groups but not in the superimposed animals where again levels were already high as a consequence of the persisting worms from the original infection. No marked changes in Paneth cell numbers were recorded but eosinophil responses were marked in both cases, although eosinophils were twice as hight as in animals infected for the first time. The significance of these results is discussed in detailed in the context of our understanding of the immunology of hookworm infections.

4.2 – INTRODUCTION

It is widely recognised that human hookworm infections play an important role in the health of populations in endemic regions and that they also have a significant impact on local economies (Banwell and Schad, 1978; Gilles, 1985). The chronicity of the disease caused by hookworms is not disputed but the existence of acquired immunity in humans is still controversial (Behnke, 1987a). In several experiments involving experimental infection of human volunteers, no signs of protective immunity in the host were evident (Ball and Bartlett, 1969; Behnke, 1987b; Hotez *et al.*, 1996).

On the other hand, experiments with non-human hookworms, such as those of dogs furnished evidence of resistance and acquired immunity (Carroll and Grove, 1985a and b). However, compared with some intestinal nematodes, such as *T. spiralis* and *N. brasiliensis,* hosts are slow to acquire resistance against reinfection with hookworms. The total egg counts from dogs repeatedly infected with *A. caninum* indicated that infected animals do not become totally resistant to reinfection as some adult worms still managed to develop successfully in challenged dogs (Otto and Kerr, 1939). There was, however, convincing evidence that dogs infected with hookworms did develop some resistance to infection (Miller, 1967). Young pups are susceptible to infection with *A. caninum*, becoming resistance by 11 months of age, as reflected by the establishment of adult worms (Miller, 1965a). Carroll and his colleagues (1985b) also concluded that age resistance is an important factor in the host-parasite relationship of the species *A. ceylanicum*. Moreover, preliminary data from hamsters infected with *A. ceylanicum* supported the idea that immunity to this particular hookworm exists (Behnke *et al.*, 1997; Gupta and Katiyar, 1985; Menon and Bhopale, 1985b).

Cellular changes in the host intestine associated with the presence of hookworms during primary infection and in immune challenged animals were also reported by Garside *et al.*, (1990). It was found that marked mastocytosis occurred in the immune-challenged group of hamsters, with fewer mast cells in challenge-control animals that were experiencing infection for the first time

(Garside *et al.*, 1990). Mast cell counts were elevated in both naive-challenged and immune-challenged groups compared with naïve groups. These changes were found to be dependent upon the length of primary exposure to the parasite with longer primary infections resulting in lower secondary worm burdens, more intense antibody responses and increase in mast and goblet cell numbers (Garside *et al.*, 1990).

Goblet cells are believed to play an important role in mucosal immunity (Miller, 1984). Their responses were detected during hookworm infection in the immune challenged hamsters by Garside (1990) and it was surprising to find that goblet cell responses were reduced in immune-challenged hamsters. It was suggested that the reduction in goblet cell responses in the immune challenged hamsters was probably related to the early expulsion of the worms.

Some studies have observed changes in eosinophil numbers in the blood and in the intestine of mice during ancylostomosis. These studies indicated that eosinophilia in multiple infections is an anamnestic response (Gowri and Vardhani, 1992; Vardhani, 2003). In this chapter, in addition to monitor tissue eosinophil number, the Paneth cell are studied for the first time in hamsters exposed to challenge infection with *A. ceylanicum*. Two experimental protocols are exploited. In the first, the primary immunising worm burden was removed prior to the challenge and in the second, the challenge infection was superimposed on the primary infection. The aim of these experiments was to identify and quantify the cellular changes occurring in the intestinal mucosa during these two experimental combinations.

4.3 – EXPERIMENTAL DESIGN AND RESULTS

Two separate experiments were carried out at different times using *A. ceylanicum* to observe the changes in the intestinal mucosa and the mucosal cellular responses in the intestine during the secondary response in hamsters. Both experiments comprised five different treatments, three of which were similar in both experiment.

4.3.1- Challenge infection with *A.ceylanicum*

This experiment comprised two groups of naïve-uninfected hamsters, which were killed on days 73 and 94 respectively. Another two groups that received primary infection of 50 L3 *A. ceylanicum* were killed on days 73 and 94. A further two groups that received primary infection of 50 L3 *A. ceylanicum* were treated with Ivermectin on day 35 (Note the similarity to the recovery experiment as explained in chapter three, Section 3.4.4) and were also killed on day 73 and 94. Two more groups received Ivermectin on day 35 and were infected with 50 L3 *A. ceylanicum* on day 63 and then killed on day 73 and 94. Finally, four groups of five hamsters each received a primary infection with 50 L3, were then treated with Ivermectin on day 35, and then re-infected with 50 L3 again on day 63. These groups were killed on days 73, 80, 87 and 94 respectively. The data from these experiments are summarised in Table 4.1.

4.3.1.1 – Total worm recovery

The worm recovery data shown in Figure 4.1 revealed that infection established normally in primary infected hamsters. Analysis by 2-way ANOVA following the transformation [log (X+1)] of data showed that the differences in the number of worms established in infected groups (Primary, primary-challenged and naïve-challenged groups) was significantly affected by treatment ($F_{2,32}$=4.491, P<0.05) and time ($F_{3,32}$=3.310, P=0.05) but there was no significant interaction between time and treatment ($F_{2,32}$=1.829, P=0.177). It was found that there was only a small reduction in the number of worms recovered from primary infected hamsters between days 73 and 94, which was as expected. However, this reduction was not significant because of the wide variation between the groups on these days (U=8.5 n1n2=5,5 P=0.401). The naïve-challenged group had relatively high worm burdens on day 73, 10 days after infection (10.2 ± 1.7). At this time, the mean worm burden in the immune-challenged animals was much lower (2.4 ± 1), although on the following days, it fluctuated within a range of (1 – 3) worms.

Table 4.1 – Experiment 1. Experimental design and worm recovery in an experiment to assess the effect of an abbreviated primary infection with A. ceylanicum followed by challenge infection.

Treatment code	No. of hamsters	Dose of L3 given @ primary	Dose of L3 given @ secondary	Mean no. of worms recovered	Day killed
1	5	None	None	Nil	73
	5	None	None	Nil	94
2	5	50 L3	None	4.6 ± 1.806	73
	5	50 L3	None	2.8 ± 1.393	94
3	5	50 L3	None	Nil	73
	5	50 L3	None	Nil	94
4	5	None*	50 L3	10.2 ± 1.743	73
	5	None*	50 L3	4.2 ± 1.158	94
5	5	50 L3*	50 L3	2.4 ± 1.030	73
	5	50 L3*	50 L3	0.4 ± 0.4	80
	5	50 L3*	50 L3	3 ± 1.414	87
	5	50 L3*	50 L3	2.6 ± 0.245	94

Treatment code key:

1- Received nothing (naïve).
2- Received primary infection only on day 0 (Primary).
3- Received primary infection on day 0 then Ivermectin treatment on day 35 (abbreviated primary)
4- Received infection of 50 L3 *A. ceylanicum* on day 63 only (naïve-challenged infection).
5- Received a secondary infection of 50 L3 *A. ceylanicum* on day 63 following the abbreviated initial infection (abbreviated primary – challenged infection).

* Note: Groups C, D, G, I, H, E, J and F were given Ivermectin orally on day 35 following initial infection.

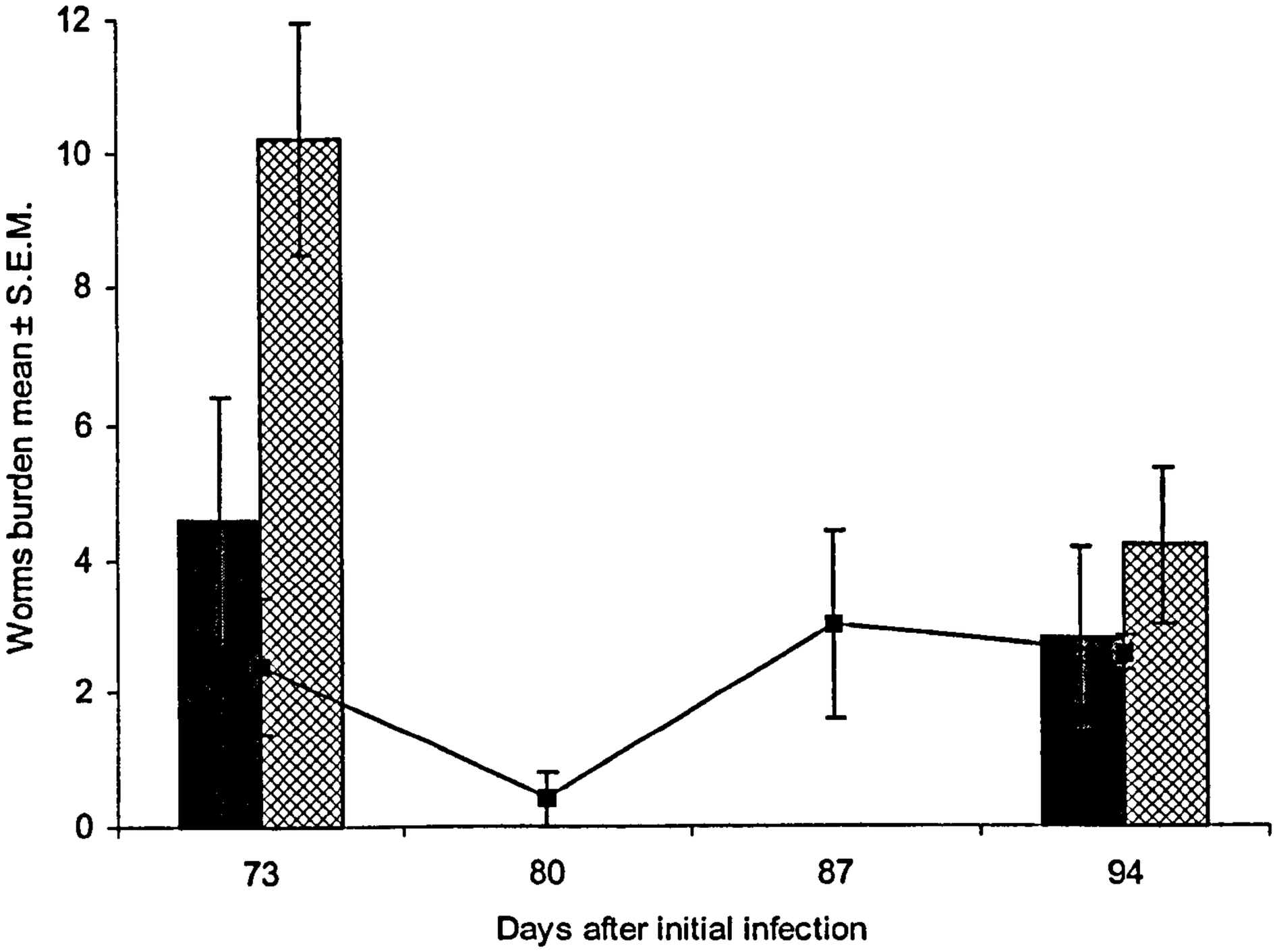


Figure 4.1 – Mean number of adult *A. ceylanicum* worms (± S.E.M.) recovered from the small intestine of:

Primary infected hamsters (▬).

Naïve–challenged hamsters (⊠).

Abbreviated primary-challenged hamsters (—■—).

<u>Statistical analysis:</u>

Analysis of transformed [log (X+1)] data by 2-way ANOVA with treatment (3 level, primary-not challenged, primary-challenged and naïve-challenge) and time (4 level, 73, 80, 87 and 94 days after initial infection on day 0) revealed:

Main effect of treatment	($F_{2,32}$=4.491, P<0.019)
Main effect of time	($F_{3,32}$=3.310, P=0.032)
Interaction between time and treatment	($F_{2,32}$=1.829, P=0.177)

Model R^2=0.378

4.3.1.2 – Assessment of intestinal pathology

Samples fixed in Clark's fixative (See 2.4.1 and appendix) and stained with Schiff's reagent were observed and analysed to measure changes in the height of the villi and the depth of the crypts during the course of primary and secondary infection with *A. ceylanicum* in DSN golden hamsters. As can be seen from figure 4.2, villous height remained relatively high in the intestines of the naïve-uninfected hamsters, ranging between 753-812.8 µm. Villous height was either normal or slightly higher in infected hamsters treated with Ivermectin compared to naïve animals. On the other hand, the villous height was at its shortest and remained as such, around 57.3 µm and 64.3 µm, on days 73 and 94 respectively, in primary infected hamsters. Nevertheless, naïve-challenged groups of hamsters showed a reduction in villous height by half, ranging from 404.5 ± 25.8 on day 73, to 430.3 ± 14.2 on day 94. Analysis by 2-way ANOVA of the data from this experiment showed a significant main effect of time ($F_{3,47}$=15.588, P<0.001) and treatment ($F_{4,47}$=159.917, P<0.001) on villous height, and a significant interaction between treatment and time ($F_{4,47}$=48.074, P<0.001).

Crypt depth was also affected by the type of infection, showing different response patterns. As can be seen from the graph, crypt depth remained relatively shallow and consistent, ranging between 148 µm and 172.5 µm in both naïve and abbreviated primary groups of hamsters. However, this depth increased in primary groups, ranging 494.3 µm ± 24.9 on day 73, and 515.9 ± 39.6 on day 94. Analysis of crypt depth data from abbreviated primary – challenged groups showed that crypt depth increased significantly with time over the period of this experiment (r_s=0.926, n=19, P<0.001). The crypt depth increased from 169.5 µm ± 8.1 on day 73 up to 649.4 µm ± 47.8 on day 94 during the abbreviated primary – challenged, and from 213.2 µm ± 14.6 on day 73 to 357.2 µm ± 30.9 on day 94 in naïve – challenged groups. When the data were analysed by 2-way ANOVA, crypt depth was seen to have changed significantly with time and treatment and there was a highly significant interaction between time and treatment ($F_{3.47}$=23.217, P<0.001; $F_{4.47}$=51.706, P<0.001; and $F_{4.47}$=20.913, P<0.001, respectively).

Figure 4.2 – Mean villous height + (±SEM) and mean crypt depths - (± SEM) measured in the intestine of:

Naïve-uninfected hamsters ( )

Primary infected hamsters ( ).

Abbreviated primary hamsters ( ).

Naïve-challenged hamsters ( ).

Abbreviated primary-challenged hamsters ( ).

Statistical analysis:

Analysis of these data by 2-way ANOVA with treatment (5 level, naïve-not challenged, primary-not challenged, primary-challenge, naïve-challenge and abbreviated primary-not challenged) and time (4 level, 73, 80, 87 and 94 days after initial infection on day 0) revealed:

Main effect of treatment on villi ($F_{4,47}$=159.917, P<0.001)

Main effect of time on villi ($F_{3,47}$=15.588, P<0.001)

Interaction between time and treatment on villi ($F_{4,47}$=48.074, P<0.001)

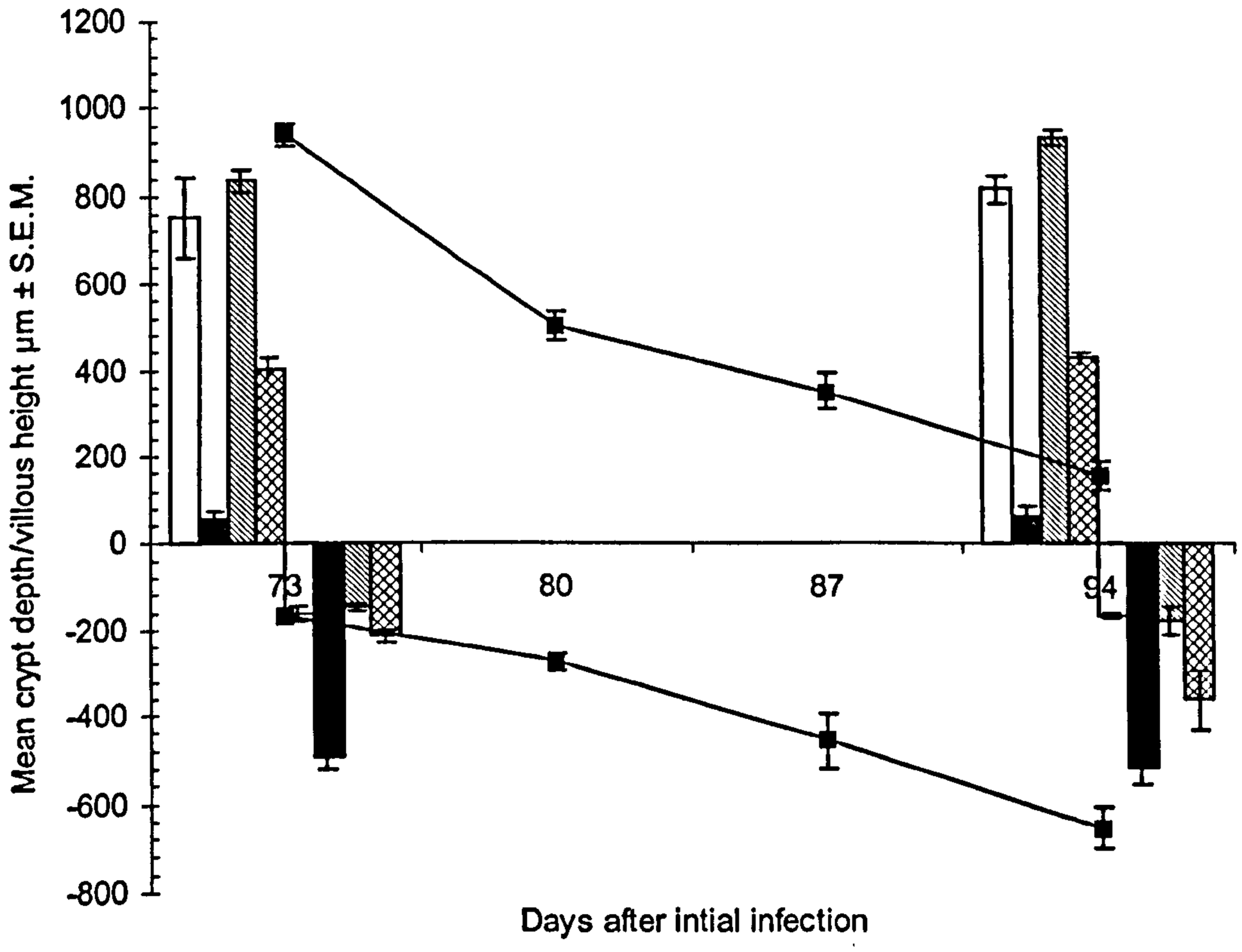
Model R^2=0.938

Main effect of treatment on crypts ($F_{4,47}$=51.706, P<0.001)

Main effect of time on crypts ($F_{3,47}$=23.217, P<0.001)

Interaction between time and treatment on crypts ($F_{4,47}$=20.913, P<0.001)

Model R^2=0.855


1200
1000
800
600
400
200
0
-200
-400
-600
-800
Mean crypt depth/villous height µm ± S.E.M.
73
80
87
94
Days after intial infection

4.3.1.3 – Cellular division in the crypt of Lieberkuhn

Figure 4.3 shows that the number of mitotic figures in the various groups of hamsters subjected to different treatments, including naïve, abbreviated primary, naïve-challenged and primary groups of hamsters, over the time of the experiment were low and similar, ranging between approximately 8 and 10 figures/crypt and exhibiting no marked change over time. However, analysis of the data by 2-way ANOVA showed that there was a main effect of time on the number of mitotic figures ($F_{3,47}$= 10.527, P<0.001) and treatment ($F_{4,47}$=30.772, P<0.001) and a highly significant interaction between time and treatment ($F_{4,47}$= 16.565, P<0.001). The number of mitotic figures in abbreviated primary-challenged hamsters was found to be relatively low and at the same level as mitotic figures in other treatments over the first 24 days. They then increased to reach their highest mean of 21.5 ± 0.2 mitotic figures on day 94.

4.3.1.4 – Mast cell responses

The average mast cell numbers/mm² of tissue in the intestine of naïve hamsters had a range of 10.6 ± 0.9 cells/mm² on day 73 and 11.7 ± 0.9 cells/mm² on day 94, similar figures to those found in previous experiments as presented in earlier chapters. Figure 4.4 shows that mast cell numbers remained low throughout in naïve groups with no marked differences between groups killed at different times. This number rose up to a mean of 55.4 cells/mm² on day 73 and 49.8 cells/mm² on day 94 in primary hamsters. Nevertheless, the numbers of mast cells over the period of this experiment in primary hamsters remained relatively stable. Moreover, analysis of data from the abbreviated primary – challenged groups revealed that there was no significant change in mast cell number with time (r_s=0.419, n=20, P=0.066). In contrast, mast cell numbers were found to increase in naïve-challenged groups where the number rose from 11.9 ± 1.2 cells/mm^2 on day 73 to a mean of 33.7 ± 4.7 cells/mm^2 on day 94. These changes are illustrated in the figure and, as can be seen, whereas there was slight reduction from 12.0 cells/mm² ± 0.7 on day 73, to 8.78 cells/mm² ± 0.99 on day 94 in the abbreviated primary groups,

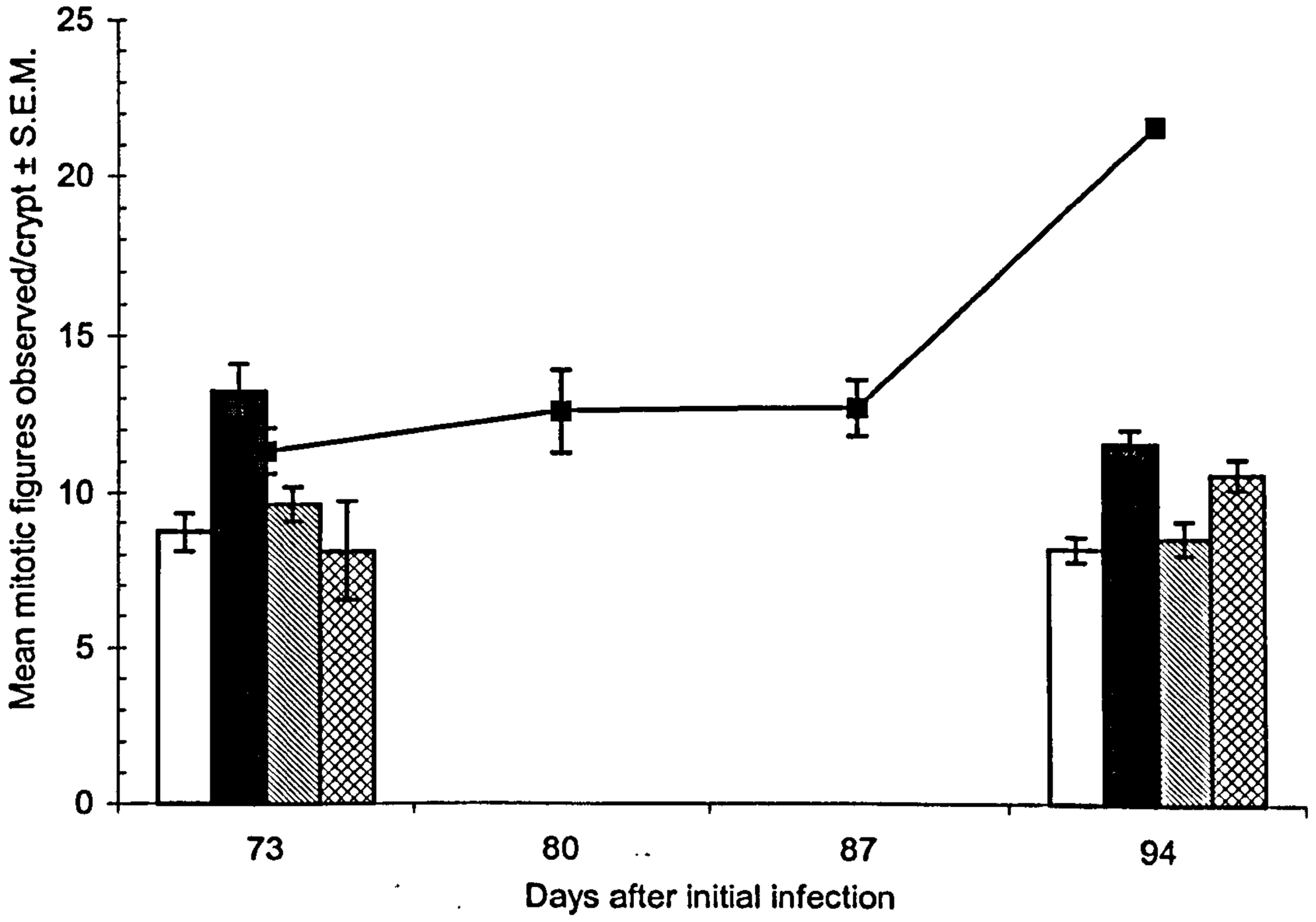


Figure 4.3 – Mean number of mitotic figures observed in the Crypt of Lieberkuhn in the intestine of:

Naïve-uninfected hamsters (▭).

Primary infected hamsters (▩).

Abbreviated primary hamsters (▨).

Naïve-challenged hamsters (▩).

Abbreviated primary-challenged hamsters (—■—).

Statistical analysis:

Analysis of these data by 2-way ANOVA with treatment (5 level, naïve-not challenged, primary-not challenged, abbreviated primary-challenged, naïve-challenge and abbreviated primary-not challenged) and time (4 level, 73, 80, 87 and 94 days after initial infection on day 0) revealed:

Main effect of treatment ($F_{4,47}$=30.772, P<0.001)

Main effect of time ($F_{3,47}$=10.527, P<0.001)

Interaction between time and treatment ($F_{4,47}$=16.565, P<0.001)

Model R^2=0.757

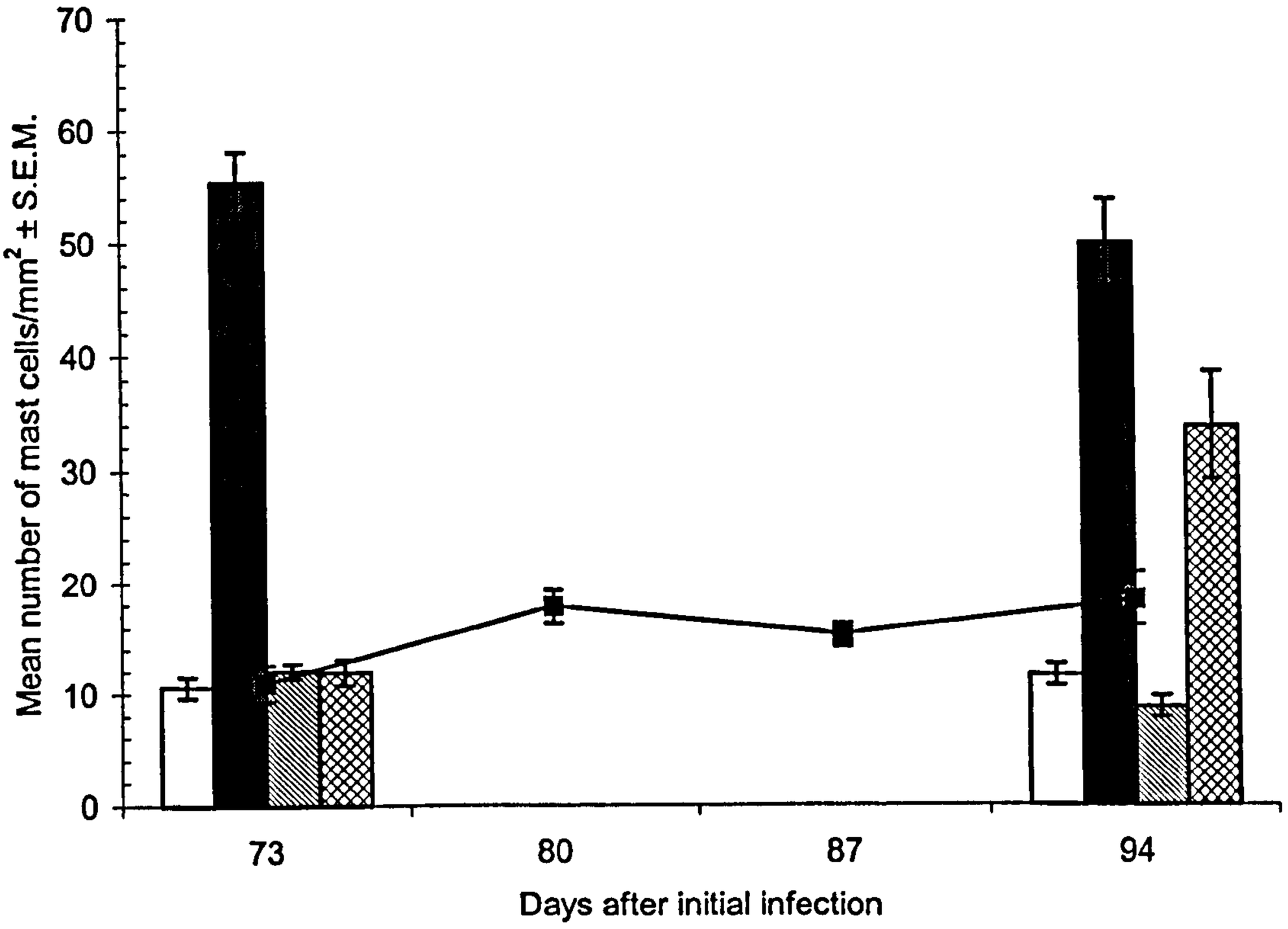


Figure 4.4 – Mean number of mast cells/mm² (± S.E.M.) of intestinal tissue in:

Naïve-uninfected hamsters (▭).

Primary infected hamsters (▬).

Abbreviated primary hamsters (▨).

Naïve-challenged hamsters (▩).

Abbreviated primary-challenged hamsters (—■—).

Statistical analysis:

Analysis of these data by 2-way ANOVA with treatment (5 level, naïve-not challenged, primary-not challenged, abbreviated primary-challenged, naïve-challenged and abbreviated primary-not challenged) and time (4 level, 73, 80, 87 and 94 days after initial infection on day 0) revealed:

Main effect of treatment	($F_{4,48}$=123.288, P<0.001)
Main effect of time	($F_{3,48}$=3.514, P<0.05)
Interaction between time and treatment	($F_{4,47}$=11.960, P<0.001)

Model R^2=0.903

there was a marked increase from 11.9 cells/mm² ± 1.2 on day 73, to 33.688 cells/mm² ± 4.7 on day 94 in the naïve-challenged group. Analysis by 2-way ANOVA revealed a significant main effect of time ($F_{3,48}$=3.514, P<0.05) and more importantly, a highly significant effect of treatment ($F_{4,48}$=123.288, P<0.001) and interaction between time and treatment ($F_{4,48}$=11.960, P<0.001).

4.3.1.5 – Goblet cell responses

Goblet cells were again assessed during this experiment and the results are summarised in Figure 4.5. A significant main effect of time and treatment was highlighted when 2-way ANOVA was used (main effect of time, $F_{3,48}$=21.006, P<0.001 and treatment, $F_{4,48}$=13.234, P<0.001). Furthermore, there was a significant interaction between time and treatment ($F_{4,48}$=3.358, P<0.05). It was found that the count for all the groups was elevated at different stages in relation to those for naïve animals (38-45 cells/mm²). However, hamsters treated with anthelmintic (abbreviated primary group) showed similar goblet cell numbers to those in naïve animals with a range between 52 and 54 cells/mm² on days 73 and 94. All other treatments showed an increase in the number of goblet cells over the period of the experiment with the exception of a decline from day 87 towards the end of this experiment in abbreviated primary-challenged hamsters. The correlation test showed that there was a significant increase in numbers of goblet cells during the course of the primary-challenged treatment (r_s=0.589, n=20, P<0.01) when the number increased from 56.7 ± 5 cells/mm² on day 73 to 213.6 ± 11.9 cells/mm² on day 87, with a sharp decline to 136.9 ± 22.6 cells/mm² towards the end of this experiment. Differences in goblet cells were slight in primary naïve-challenged groups over the course of this experiment. The number rose from 105.6 ± 11.9 cells/mm^2 on day 73 to 162.1 ± 28.7 cells/mm^2 on day 94 in primary hamsters and from 71.7 ± 10.7 cells/mm^2 on day 73 to 95.2 ± 18.4 cells/mm^2 on day 94 in the naïve-challenged group.

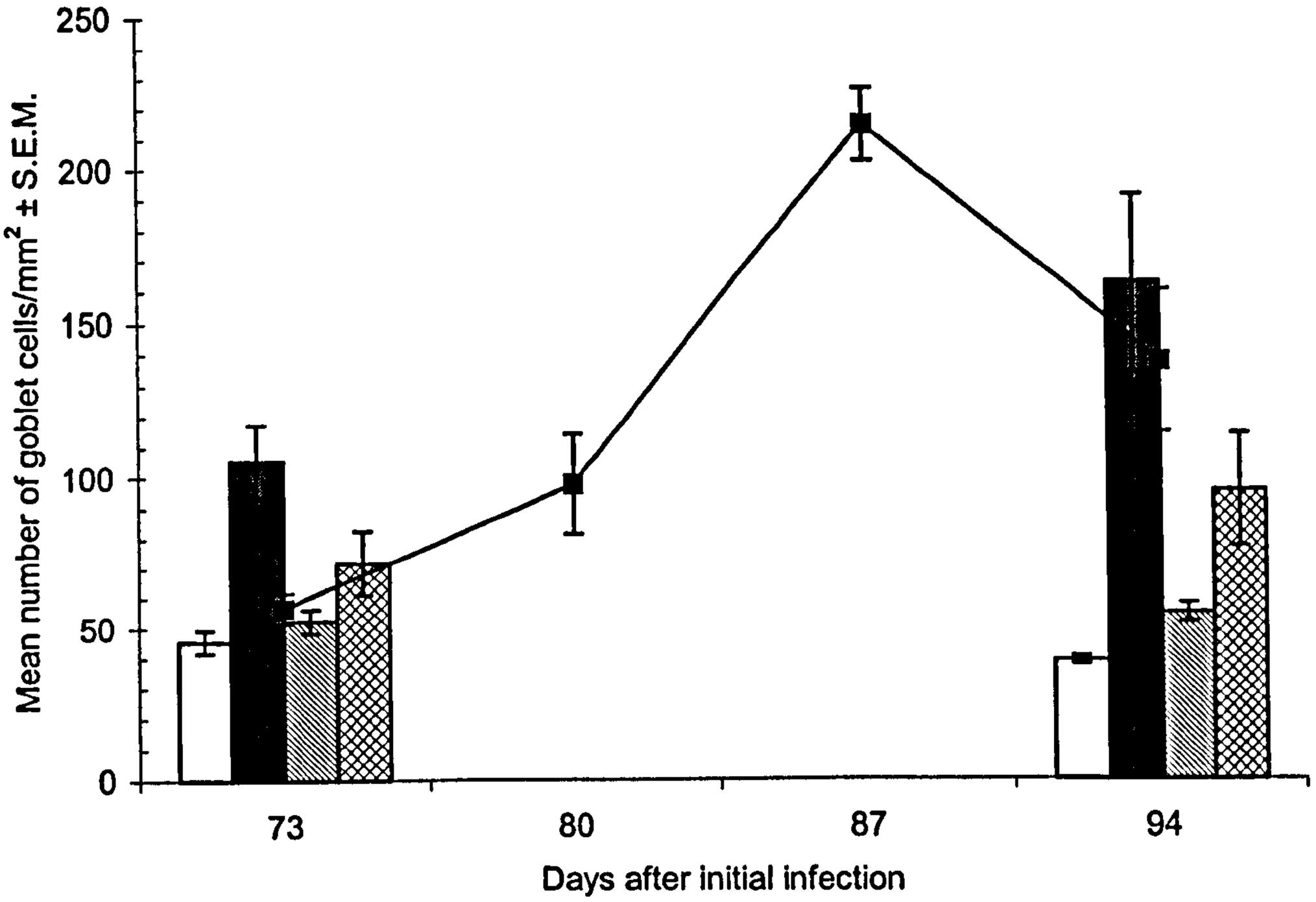


Figure 4.5 – Mean number of goblet cells/mm² (± S.E.M.) of the intestinal tissue in:

Naïve-uninfected hamsters (▭).

Primary infected hamsters (▬).

Abbreviated primary hamsters (▨).

Naïve-challenged hamsters (▩).

Abbreviated primary-challenged hamsters (—■—).

<u>Statistical Analysis:</u>

Analysis of these data by 2-way ANOVA with treatment (5 level, naïve-not challenged, primary-not challenged, abbreviated primary-challenge, naïve-challenge and abbreviated primary-not challenged) and time (4 level, 73, 80, 87 and 94 days after initial infection on day 0) revealed:

Main effect of treatment ($F_{4,48}$=13.234, P<0.001)

Main effect of time ($F_{3,48}$=21.006, P<0.001)

Interaction between time and treatment ($F_{4,47}$=3.358, P<0.001)

Model R^2=0.713

4.3.1.6 – Paneth cell responses

Phloxine-tartrazine stained cells in the crypts of Lieberkuhn were counted to assess the change in Paneth cell numbers during infection with *A. ceylanicum* in hamsters. As can be seen from figure 4.6 the number of Paneth cells remained as high as 2 cells/crypt on days 73 and 94 in naïve groups with no marked changes in the mean number of cells. Analysis by 2-way ANOVA showed that there was significant main effect of treatment on Paneth cells ($F_{4,47}$=25.568, P<0.001). The effect of time, however, was not significant ($F_{3,47}$=2.500, P=0.071) and no significant interaction between time and treatment ($F_{4,47}$=1.259, P=0.299) was found. Paneth cell numbers were much lower in the primary groups compared to all other treatments with a number of 1 ± 0.2 cells/crypt on day 73 declining to 0.7 ± 0.2 cells/crypt on day 94. On the other hand, Paneth cell numbers were at their highest (4-6 cells/crypt) following worm removal using an anthelmintic drug on day 35. Furthermore, neither abbreviated primary-challenged hamsters nor naïve-challenged groups showed significant changes in Paneth cells during the course of this experiment, (r_s=-0.008, n=19, P=0.974and r_s=-0.592, n=10, P=0.071), which might be due to the reduction in the mean of Paneth cells numbers between days 80 and 87 in the former and wide error variation in both the former and the latter.

4.3.1.7 – Eosinophil responses

The number of eosinophils in the intestinal tissue of hamsters were counted and assessed. Figure 4.7 shows the variation in eosinophil levels in hamsters' intestines during the differing treatments. It is clear that the number of eosinophils increased dramatically in all hamsters with established worms compared to naïve animals and those the abbreviated primary groups. This number reached a peak of 692.1 ± 43.6 cells/mm² in the naïve-challenged group on day 94. 2-way ANOVA showed a significant main effect of time ($F_{3,45}$=11.552, P<0.001) and treatment ($F_{4,45}$=63.916, P<0.001), but no interaction between time and treatment ($F_{4,45}$=2.357, P=0.068). The level remained relatively low at around 20-30 cells/mm² in naïve groups at different

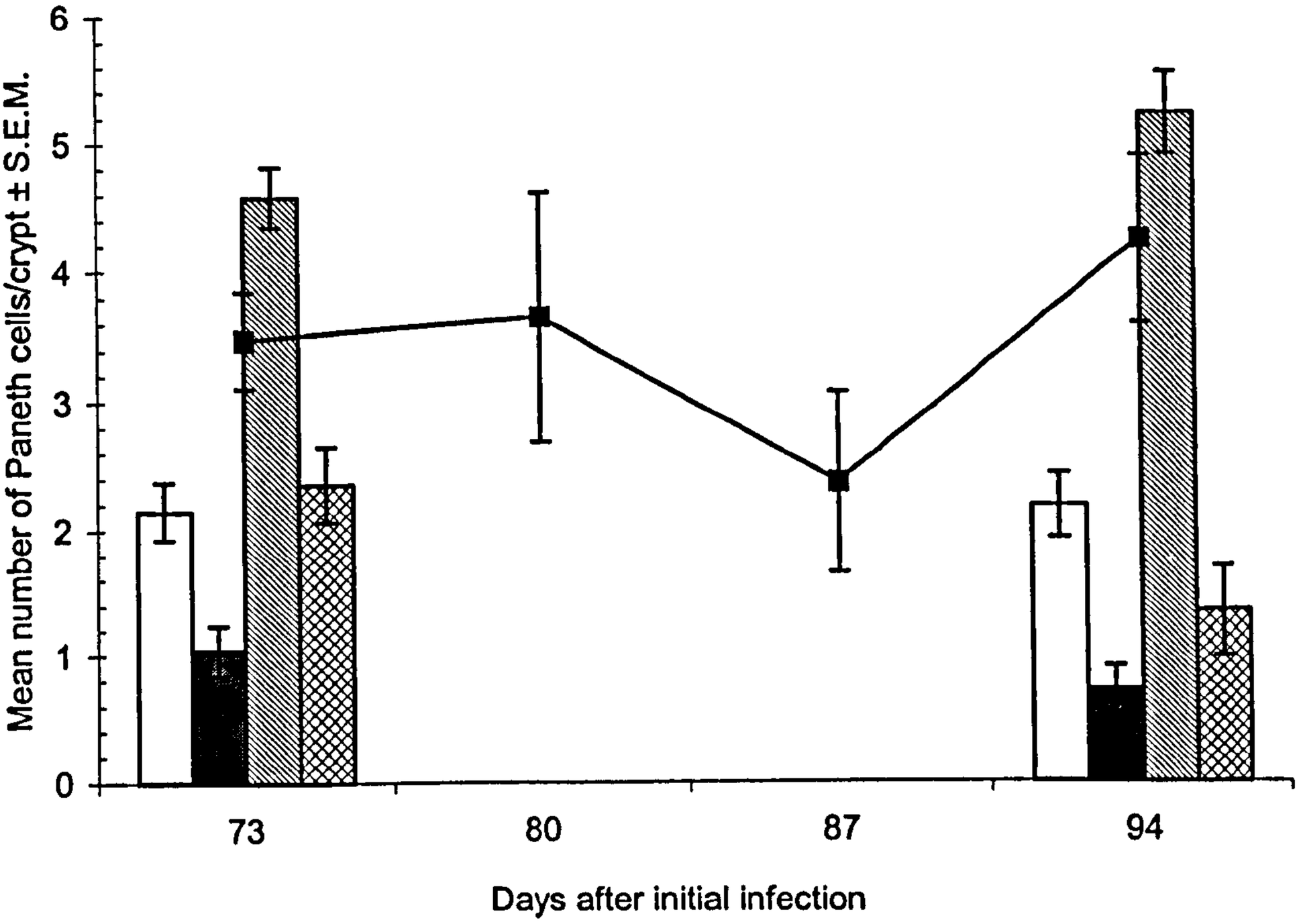


Figure 4.6 – Mean number of Paneth cells/crypt (± S.E.M.) in the intestinal tissue of:

Naïve-uninfected hamsters ( ).

Primary infected hamsters ( ).

Abbreviated primary hamsters ( ).

Naïve-challenged hamsters ( ).

Abbreviated primary-challenged hamsters ( ).

<u>Statistical analysis:</u>

Analysis of these data by 2-way ANOVA with treatment (5 level, naïve-not challenged, primary-not challenged, abbreviated primary-challenge, naïve-challenge and abbreviated primary-not challenged) and time (4 level, 73, 80, 87 and 94 days after initial infection on day 0) revealed:

Main effect of treatment ($F_{4,47}$=25.568, P<0.001)

Main effect of time ($F_{3,47}$=2.500, P=0.071)

Interaction between time and treatment ($F_{4,47}$=1.259, P=0.299)

Model R^2=0.634

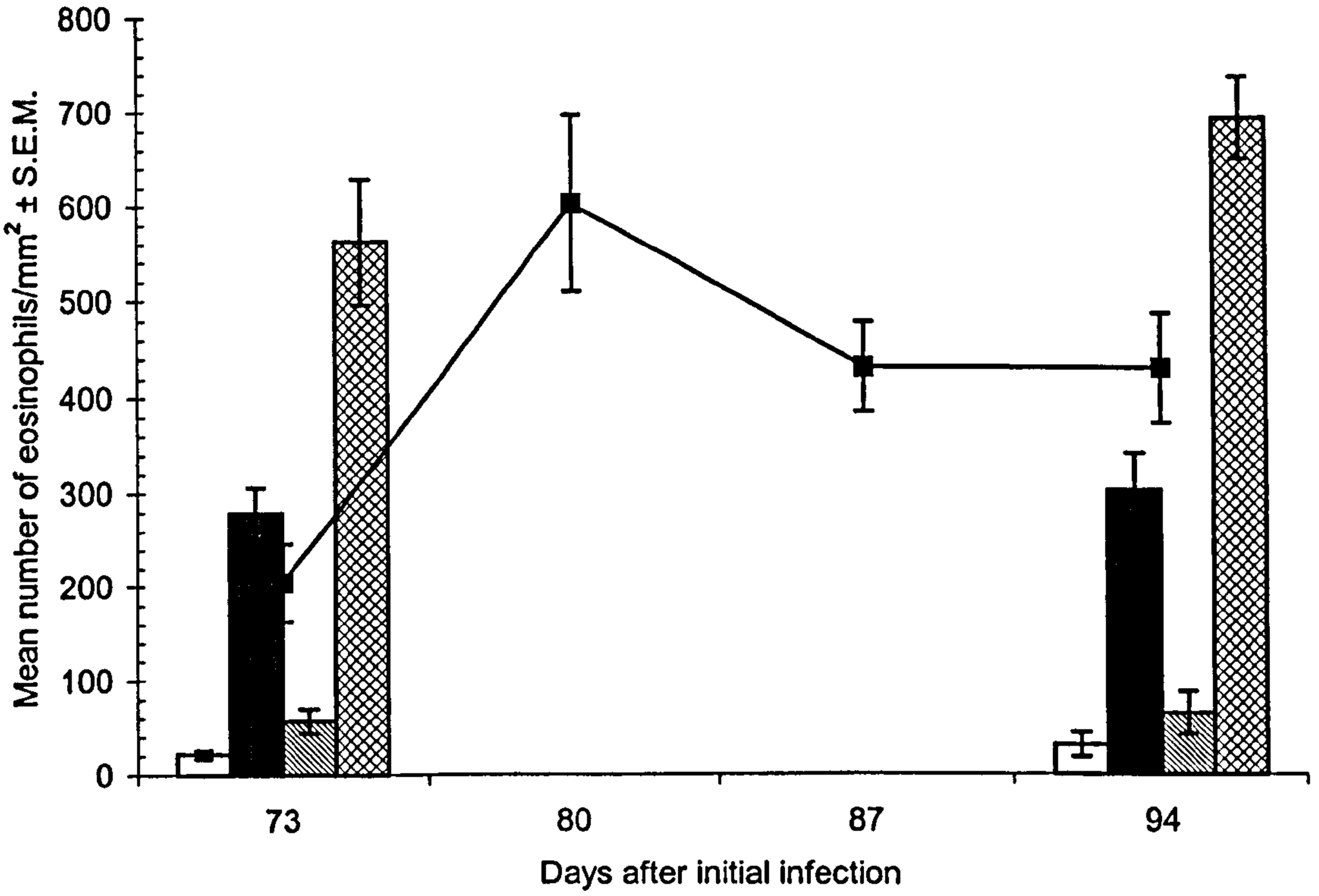


Figure 4.7 – Mean number of eosinophils/mm² (± S.E.M.) in the intestinal tissue of:

Naïve-uninfected hamsters ( ).

Primary infected hamsters ( ).

Abbreviated primary hamsters ( ).

Naïve-challenged hamsters ( ).

Abbreviated primary-challenged hamsters ( ).

<u>Statistical analysis:</u>

Analysis of these data by 2-way ANOVA with treatment (5 level, naïve-not challenged, primary-not challenged, abbreviated primary-challenged, naïve-challenged and abbreviated primary-not challenged) and time (4 level, 73, 80, 87 and 94 days after initial infection on day 0) revealed:

Main effect of treatment ($F_{4,45}$=63.916, P<0.001)

Main effect of time ($F_{3,45}$=11.552, P<0.001)

Interaction between time and treatment ($F_{4,45}$=2.357, P=0.068)

Model R^2=0.851

times with no remarkable changes observed. The abbreviated primary groups showed similar levels of eosinophils in tissue to those of naïve animals with numbers ranging from 57.2 ± 12.9 cells/mm² on day 73 to 65.5 ± 22.5 cells/mm² ± on day 94. Furthermore, there were no significant changes in eosinophils over time in abbreviated primary-challenged groups (r_s=0.441, n=18, P=0.067).

4.3.2 – Superimposed infection with *A.ceylanicum*

This experiment was carried out to examine the effect of a superimposed secondary infection with *A. ceylanicum* in hamsters that are harbouring a primary infection. The plan for this experiment included five different treatments, four of which were similar to those in the previous challenge-infection experiment. Table 4.2 summarises the experimental design. A total of 70 adult female DSN hamsters were used and were arranged under five different treatments. Three treatments, naïve, primary and naïve-challenged group resembled those in the previous challenge-infection experiment and each included two groups of five hamsters, the first group being killed on day 73 and the second on day 94 post-infection. This experiment included a fourth group comprising 4 groups of five hamsters given the primary and challenge-infection but treated after the primary to remove the initially established worms and killed on days 73, 80, 87 and 94 post-infection. In addition, another group comprising four groups of five hamsters was given a superimposed challenge-infection with 50 L3 *A. ceylanicum* on day 63 and then killed on days 73, 80, 87 and 94 post-infection.

4.3.2.1 – Total worm recovery

Changes in *A. ceylanicum* worm burdens over time following initial infection, as found from this experiment, are illustrated in Figure 4.8 (A). Different stages and/or sizes of *A. ceylanicum* recovered from superimposed infection are detailed in figure 4.8 (B). Analyses by 2-way ANOVA for

Table 4.2 – Experiment 2. Experimental design and worm recovery in an experiment to assess the effect of a superimposed challenged infection with *A. ceylanicum.*

Treatment code	No. of hamsters	Dose of L3 given @ primary	Dose of L3 given @ secondary	Mean no. of worms recovered	Day killed
1	5	None	None	Nil	73
	5	None	None	Nil	94
2	5	50 L3	None	1.8 ± 1.356	73
	5	50 L3	None	4.2 ± 2.588	94
3	5	None*	50 L3	5.4 ± 0.678	73
	5	None*	50 L3	3.2 ± 1.772	94
4	5	50 L3*	50 L3	1.2 ± 0.374	73
	5	50 L3*	50 L3	2.2 ± 0.860	73
	5	50 L3*	50 L3	2.4 ± 1.691	94
	5	50 L3*	50 L3	0.2 ± 0.2	87
5	5	50 L3	50 L3l	8.2 ± 2.818	73
	5	50 L3	50 L3	5 ± 1.517	94
	5	50 L3	50 L3	7.4 ± 2.874	80
	5	50 L3	50 L3	5.2 ± 0.860	94

Treatment code key:

1- Received no infection (naïve).
2- Received 50 L3 *A. ceylanicum* on day 0 (primary).
3- Receive primary infection on day 0 but were treated with Ivermectin on day 35 then 2nd with 50 L3 *A. ceylanicum* on day 63 (naïve-challenged).
4- Received 50 L3 *A. ceylanicum* on day 0 then treated with Ivermectin on day 35, then 2nd infection of 50 L3 *A. ceylanicum* on day 63 (abbreviated primary-challenged).
5- Received 50 L3 on day 0 then another 50 L3 *A. ceylanicum* on day 63 (Primary-superimposed challenged).

*** Note:** Groups G, H, I, J, M and N were given Ivermectin orally on day 35 following initial infection.

Figure 4.8 (A) – Mean total number of *A. ceylanicum* worms (± S.E.M.) recovered from the small intestines of:

Primary infected hamsters (▬▬).

Abbreviated primary-challenged hamsters (—■—).

Naïve-challenged hamsters (▭▭).

Primary-superimposed hamsters (·····●·····).

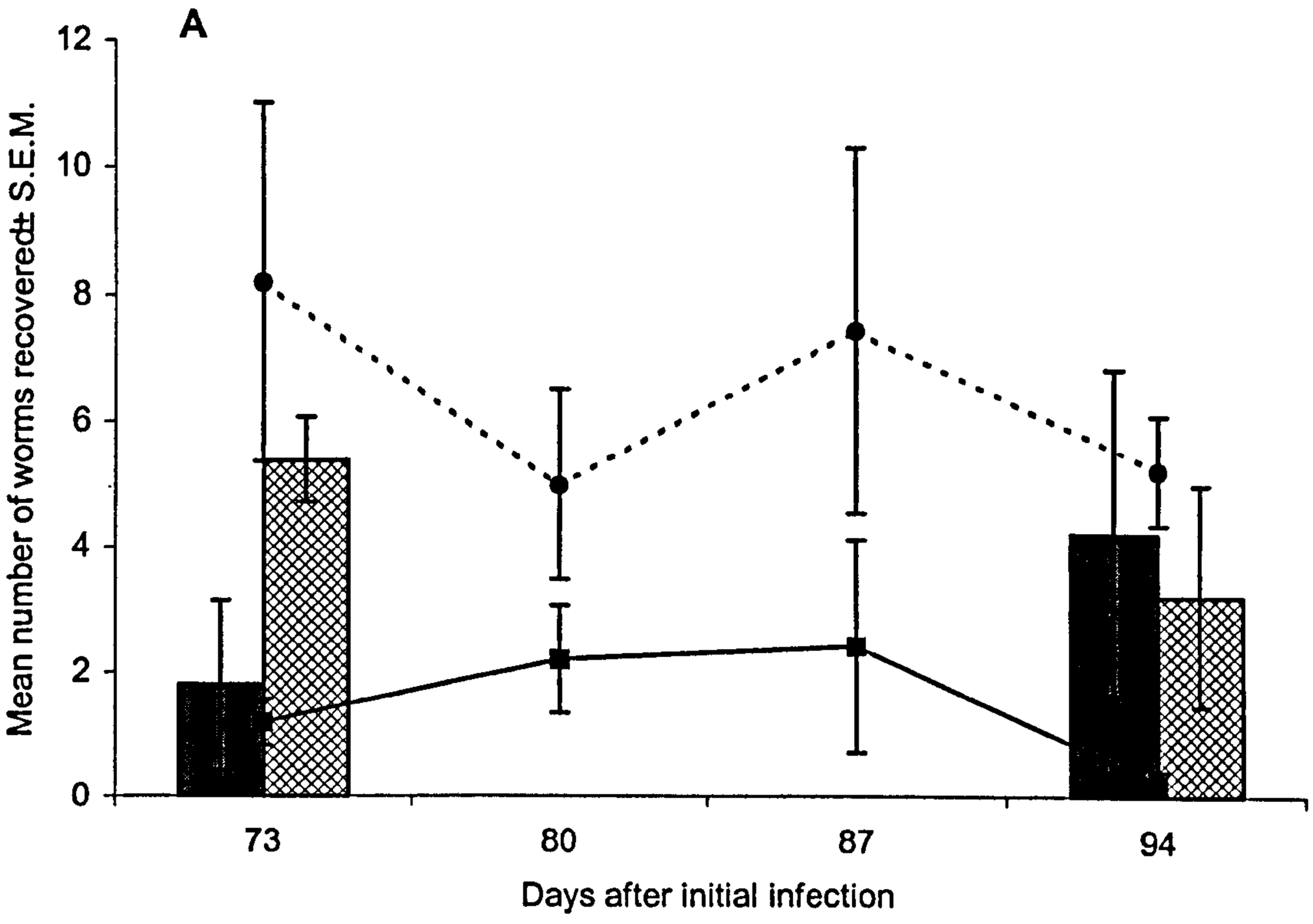
(B) – Mean L4 and small adult worms (·····●·····) compared to adult worms (·····⊙·····). Experimental details as in (A).

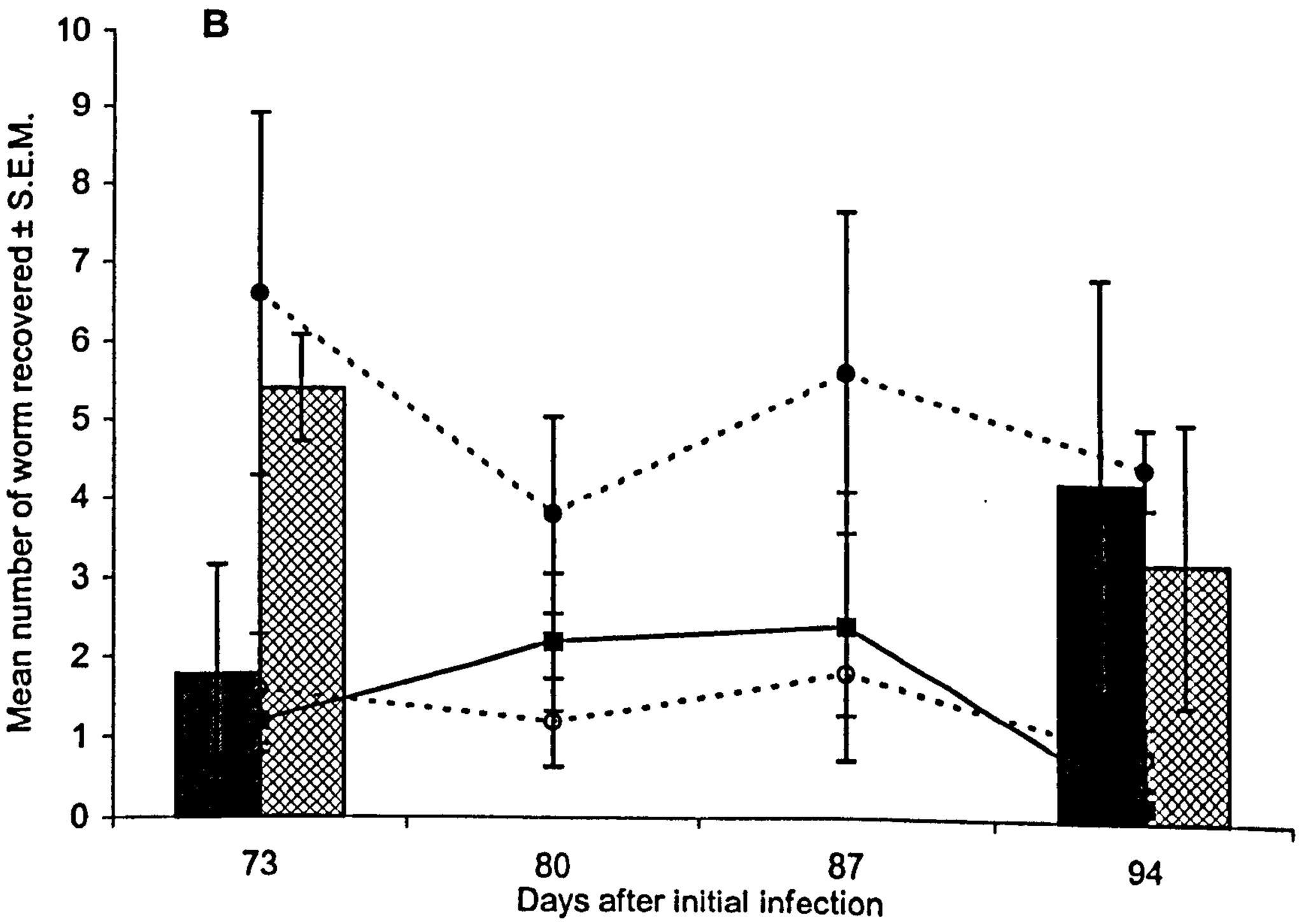
<u>Statistical analysis:</u>

Analysis of these data by 2-way ANOVA with treatment (4 level, primary-not challenged, primary-challenged, Naïve-challenged and primary-superimposed challenged) and time (4 level, 73, 80, 87 and 94 days after initial infection on day 0) revealed:

Main effect of treatment	($F_{3,48}$=10.582, P<0.001)
Main effect of time	($F_{3,48}$=0.423, P=0.737)
Interaction between time and treatment	($F_{5,48}$=2.275, P=0.062)

Model R^2=0.359


A
Mean number of worms recovered ± S.E.M.
12
10
8
6
4
2
0
73
80
87
94
Days after initial infection


B
Mean number of worm recovered ± S.E.M.
10
9
8
7
6
5
4
3
2
1
0
73
80
87
94
Days after initial infection

transformed data of worm burdens show a significant main effect of treatment ($F_{3.48}$=10.582, P<0.001). However, whereas the main effect of time was not significant ($F_{3.48}$=0.423, P=0.737), the interaction between time and treatment approaches significance ($F_{5.48}$=0.195, P=0.062). As can be seen from the figure worm burdens varied between the groups. Primary hamsters showed an increase in the mean number of worms from 1.8 ± 1.4 worms on day 73 to 4.2 ± 1.2 worms on day 94 after initial infection, which was unexpected, and contrasts with those in the previous experiments (Figure 4.1). On the other hand, naïve-challenged hamsters showed an expected reduction from the mean of 5.4 ± 0.7 worms on day 73 to 3.2 ± 1.8 worms on day 94 post infection. Both abbreviated primary-challenged and primary-superimposed challenge groups showed different mean numbers of worm recovery. The primary-superimposed challenge animals showed higher mean numbers of worms compared to those in abbreviated primary-challenged groups and, when a correlation test was used, there were no significant changes over time in either treatment (abbreviated primary-challenged, r_s=-0.397, n=20, P=0.083, and primary-superimposed challenge, r_s=-0.133, n=20, P=0.577).

4.3.2.2 – Assessment of intestinal pathology

The mean villous height (+) and crypt depth (-) illustrated in figure 4.9 for naïve-not challenged, primary-not challenged, abbreviated primary-challenged and in naïve-challenged groups approximately resembled those in the first experiment described in this chapter with a few differences in some groups. The average villous height was around 950 µm in naïve groups during the course of the experiment with heights remaining around the same levels between days 73 and 94. It was also the case that no differences were seen in villous height between days 73 and 94 in primary groups of hamsters although the heights were much shorter than those of naïve animals. Furthermore, the villous height remained at a level ranging between 451-483µm between days 73 and 94 in naïve-challenged groups. However, a continuous and significant reduction in the villous height of the abbreviated primary-challenged groups was found and the height declined from the mean of 808.8 ± 53.7 µm on day 73 to the mean of

266.3 ± 15.3 µm on day 94 (r_s=-0.869, n=20, P<0.001). On the other hand, the villous height in the primary-superimposed challenged group revealed a gradual increase from 26.5 ± 5 µm on day 73 up to 203.3 ± 72.1 µm and this was also significantly related to time (r_s=0.511, n=19, P<0.05). Analysis by 2-way ANOVA of data showed a significant main effect of time on the height of villi ($F_{3,53}$=4.086, P<0.01), a highly significant main effect of treatment ($F_{4,53}$=225.255, P<0.001) and, more importantly, a highly significant interaction between time and treatment ($F_{6,53}$=18.879, P<0.001).

The depth of crypts found in this experiment are shown in figure 4.9 and show very shallow crypts in naïve-not challenged animals with depths ranging from 136-140 µm, much as those reported in earlier chapters. The crypt depths increased from the base level of the naïve groups to reach a depth of 577 ± 33.9 µm on day 73 and remained at approximately the same level of 576 ± 50.2 µm on day 94. However, naïve-challenged groups showed an increase in the depth from 232.3 ± 13.4 µm on day 73 to 454.7 ± 38.8 µm on day 94. There was a significant increase in crypt depths from 263 ± 11.2 on day 73 to 642.3 ± 9.1 µm on day 94 in abbreviated primary-challenged groups (r_s=-0.853, n=20, P<0.001) while there a was significant reduction in primary-superimposed challenge groups from 762.3 ± 16.4 µm on day 73 to 640.5 ± 53.5 µm on day 94(r_s=-0.577, n=19, P<0.05). 2-way ANOVA showed a highly significant main effect of time and treatment on the depth of the crypt ($F_{3.53}$=11.239, P<0.001 and $F_{4.53}$=136.732, P<0.001, respectively), and a highly significant relationship between time and treatment ($F_{6.53}$=16.503, P<0.001).

4.3.2.3 – Cellular division in the crypt of Lieberkuhn

The data presented in figure 4.10 shows that the number of mitotic figures in the Crypt of Lieberkuhn was higher among infected hamsters and those undergoing various treatments compared with naïve animals, confirming the previous findings presented in earlier chapters. Analysis by 2-way ANOVA showed a significant main effect of time and treatment between groups ($F_{3.53}$=9.301, P<0.001and $F_{4.53}$=65.920, P<0.001, respectively). It was also

Figure 4.9 – Mean villous height + (±S.E.M.) and mean crypt depths - (± S.E.M.) measured in the intestine of

Naïve-uninfected hamsters (▭).

Primary infected hamsters with *A. ceylanicum* (▬).

Naïve-challenged hamsters (▭).

Abbreviated primary-challenged hamsters (—■—).

Primary-superimposed hamsters (····●····).

Statistical analysis:

Analysis of these data by 2-way ANOVA with treatment (5 level, naïve-not challenged, primary-not challenged, abbreviated primary-challenged, naïve-challenged and primary-superimposed challenged) and time (4 level, 73, 80, 87 and 94 days after initial infection on day 0) revealed:

Main effect of treatment on villi ($F_{4,53}$=225.255.917, P<0.001)

Main effect of time on villi ($F_{3,53}$=4.086, P<0.01)

Interaction between time and treatment on villi ($F_{6,53}$=18.879, P<0.001)

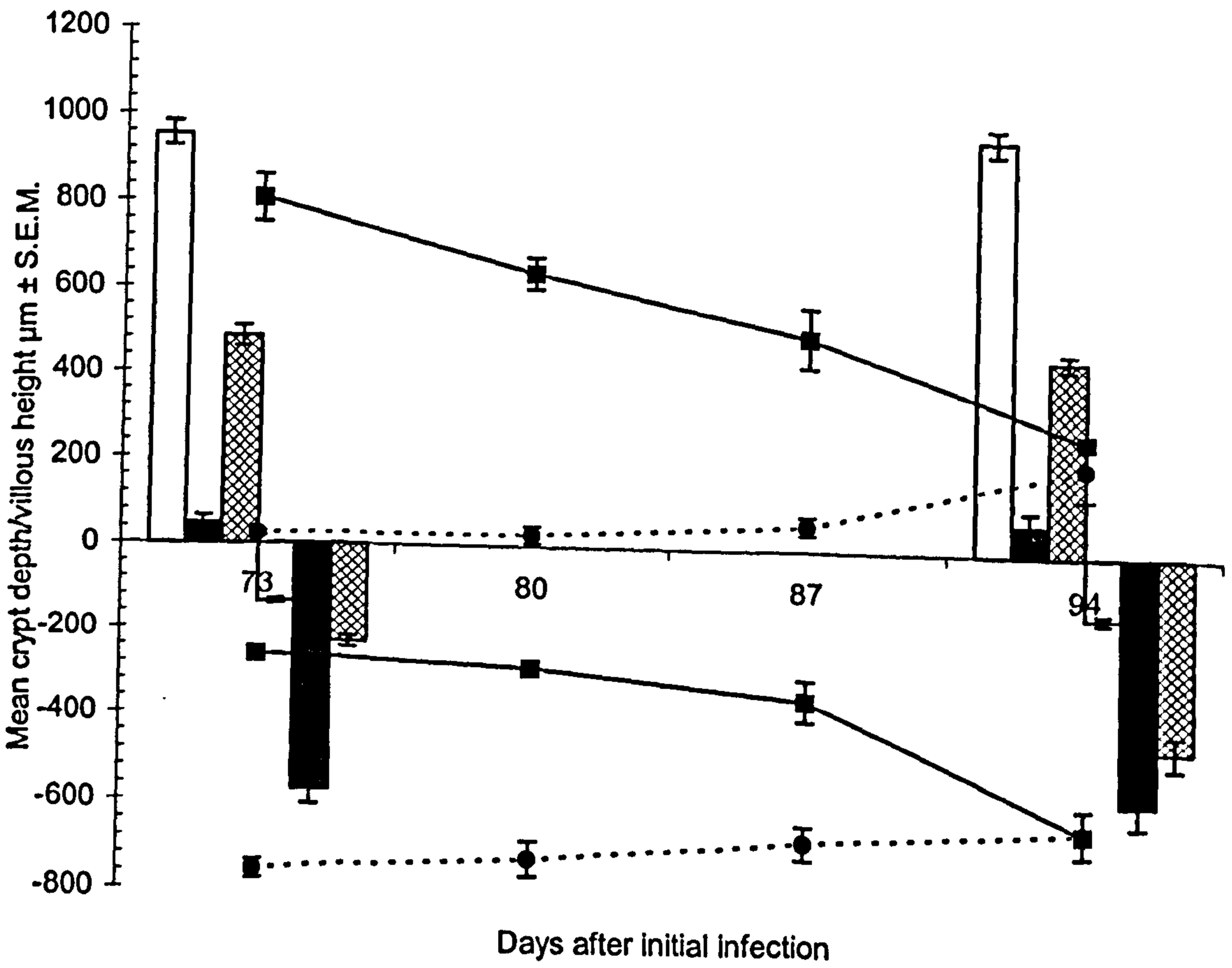
Model R^2=0.940

Main effect of treatment on crypts ($F_{4,53}$=136.732, P<0.001)

Main effect of time on crypts ($F_{3,53}$=11.239, P<0.001)

Interaction between time and treatment on crypts ($F_{6,53}$=16.503, P<0.001)

Model R^2=0.910


1200
1000
800
600
400
200
0
-200
-400
-600
-800
Mean crypt depth/villous height µm ± S.E.M.
73
80
87
94
Days after initial infection

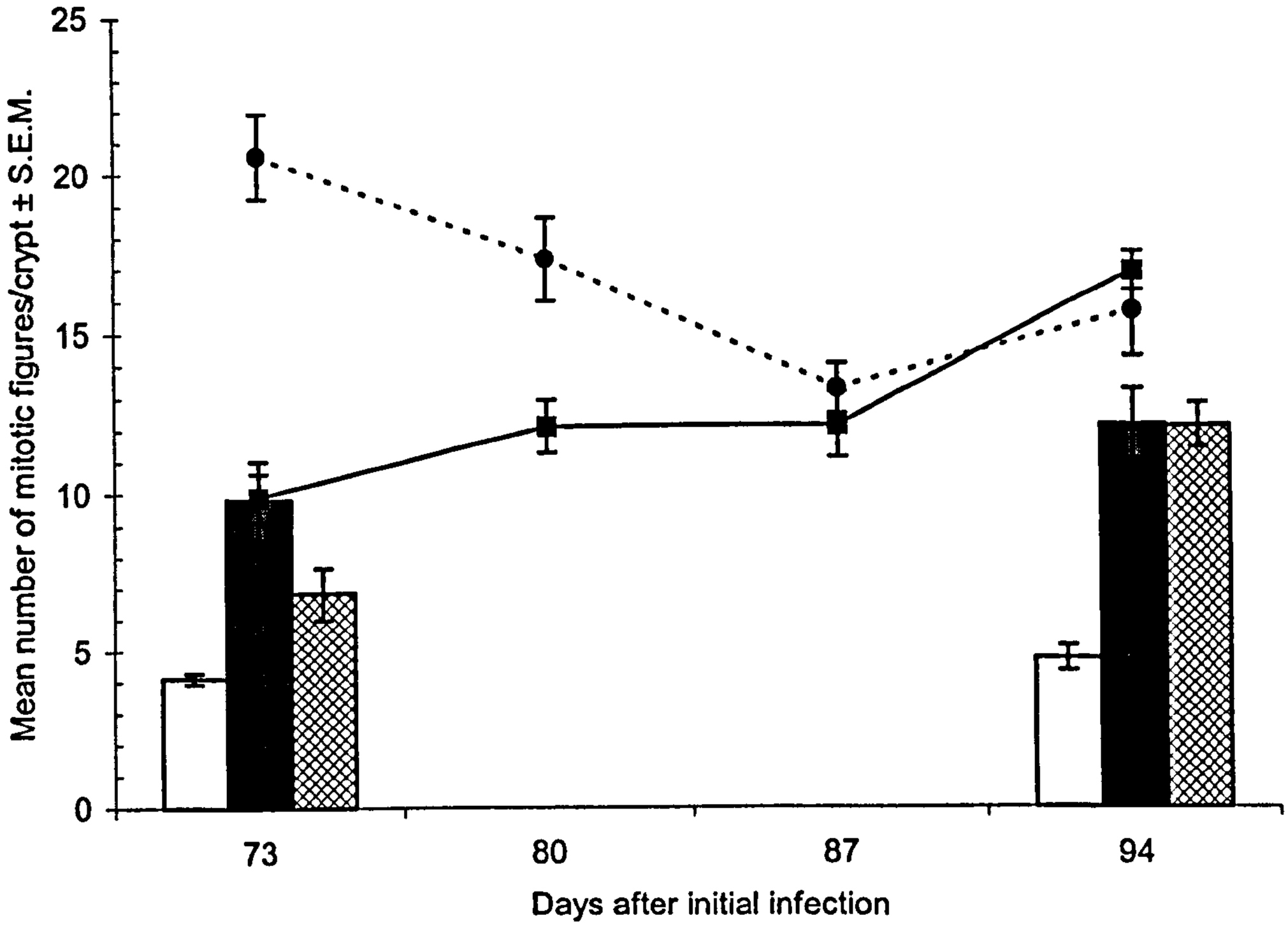


Figure 4.10 – Mean number of mitotic figures (±S.E.M.) observed in the crypt of Lieberkuhn in intestinal tissue of:

Naïve-uninfected hamsters (▭).

Primary infected hamsters with *A. ceylanicum* (▬).

Naïve-challenged hamsters with *A. ceylanicum* (▩).

Abbreviated primary-challenged hamsters (—■—).

Primary-superimposed hamsters (····●····).

<u>Statistical analysis:</u>

Analysis of these data by 2-way ANOVA with treatment (5 level, naïve-not challenged, primary-not challenged, abbreviated primary-challenged, naïve-challenged and primary-superimposed challenged) and time (4 level, 73, 80, 87 and 94 days after initial infection on day 0) revealed:

Main effect of treatment ($F_{4,53}$=65.920, P<0.001)

Main effect of time ($F_{3,53}$=9.301, P<0.001)

Interaction between time and treatment ($F_{6,53}$=10.414, P<0.001)

Model R^2=0.840

found that the interaction between treatment and time was highly significant ($F_{6.53}$=10.414, P<0.001). The number of mitotic figures was as low as 4.1 and 4.7 figures/crypt on days 73 and 94 respectively in naïve groups. However, the number increased from 9.8 ± 1.2 figures/crypt on day 73 to 12.1 ± 1.1 figures/crypt on day 94 in primary infected hamsters. Similarly, naïve-challenged groups showed an increase from 6.8 ± 0.8 on day 73 to 12.1 ± 0.7 on day 94. There was also an increase in abbreviated primary-challenged groups from 9.9 ± 0.7 on day 73 to 16.9 ± 0.6 figures/crypt on day 94. This increase was significant with regards to time (r_s=0.745, n=20, P<0.001). On the other hand, the number of mitotic figures in primary-superimposed challenged groups was relatively high on day 73 compared to other groups but declined from 21 ± 1.1 figures/crypt on day 73 to 13.2 ± 0.8 figures/crypt on day 87 with a slight increase to 15.6 ± 1.4 figures/crypt by the end of this experiment. Nevertheless, this was also significant with regards to time (r_s=-0.586, n=19, P<0.01).

4.3.2.4 – Mast cell responses

The mean number of mast cells was assessed as in Experiment 1 of this chapter and these data are illustrated in figure 4.11. It was found that there was a significant main-effect of time and treatment ($F_{3,54}$=9.869, P<0.001 and $F_{4,54}$=66.642, P<0.001, respectively) and significant interaction between treatment and time ($F_{6,54}$=9.387, P<0.001) when analysed by 2-way ANOVA. The background count of naïve animals was 10-12 mast cells/mm² of intestinal tissue and was very similar to those in experiments reported earlier. In relation to this value, all infected groups again had elevated mast cell counts. Primary groups had approximately 45-50 mast cells/mm² of intestinal tissue between days 73 and 94 and again showed no change between them. The primary-superimposed challenged groups showed a significant reduction in mast cells with time (r_s=-0.649, n=18, P<0.01) but not the abbreviated primary-challenged groups (r_s=0.155, n=20, P=0.514). Naïve-challenged hamsters showed a significant increase in mast cells with time from 11.5 mast cells/mm² on day 73 to 35 cells/mm² on day 94.

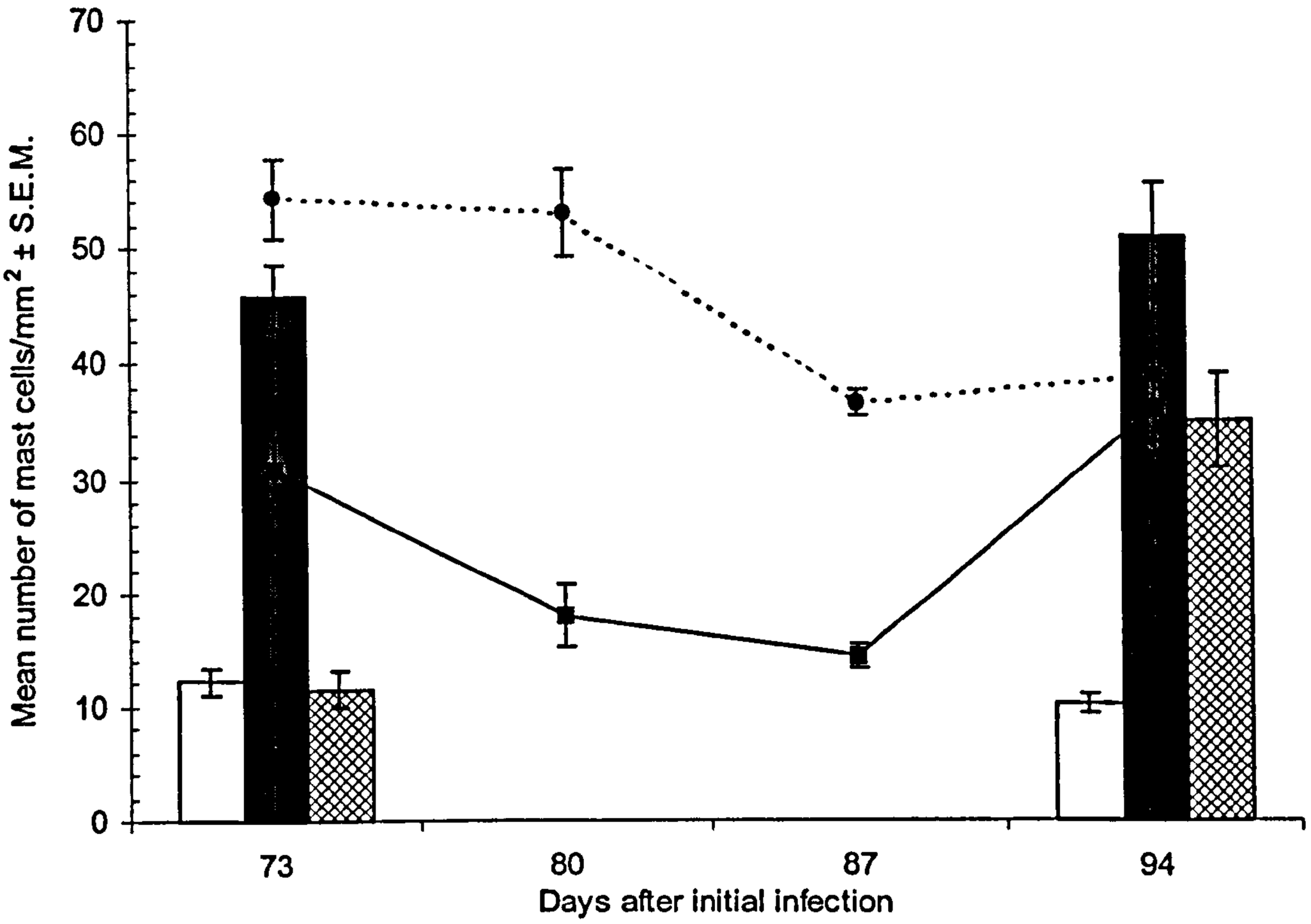


Figure 4.11 – Mean number of mast cells/mm² (± S.E.M.) of intestinal tissue in:
Naïve-uninfected hamsters (▭).
Primary infected hamsters with *A. ceylanicum* (▬).
Naïve-challenged hamsters (▨).
Abbreviated primary-challenged hamsters (—■—).
Primary-superimposed hamsters (┈┈●┈┈).

Statistical analysis:

Analysis of these data by 2-way ANOVA with treatment (5 level, naïve-not challenged, primary-not challenged, abbreviated primary-challenged, naïve-challenged and primary-superimposed challenged groups) and time (4 level, 73, 80, 87 and 94 days after initial infection on day 0) revealed:

Main effect of treatment	($F_{4,54}$=66.642, P<0.001)
Main effect of time	($F_{3,54}$=9.869, P<0.001)
Interaction between time and treatment	($F_{6,54}$=9.387, P<0.001)

Model R^2=0.827

4.3.2.5 – Goblet cell responses

Changes in goblet cells in naïve, primary and naive-challenged groups were much like those in previous experiments. As can be seen from figure 4.12 the numbers of goblet cells were greater in infected groups compared with control animals (45-49 cells/mm²). 2-way ANOVA showed that there was a main effect of time ($F_{3,53}$=3.642, P<0.05) and treatment ($F_{4,53}$=8.093, P<0.001) but there was no interaction between treatment and time ($F_{6,53}$=0.944, P=0.472). All infected groups showed an increase in goblet cell counts compared to those reported in naïve animals and these numbers increased with the passage of time. Correlation of analysis of goblet cells from primary-challenged and superimposed groups showed that no significant differences were found in the means of goblet cell counts during the course of this experiment. This might have been due to the wide variation within each group (r_s=0.047, n=20, P=0.846 and r_s=0.263, n=17, P=0.308, respectively).

4.3.2.6 – Paneth cell responses

In this experiment, Paneth cells numbers in naïve hamsters were approximately double those found in primary animals and naïve-challenged groups and were as expected, confirming earlier findings. Large variation within groups could also be seen in most of the groups, as shown in figure 4.13. Analysis of the effect of time and the interaction between time and treatment on Paneth cell number by 2-way ANOVA indicated that there was no significant main-effect ($F_{3,53}$=1.339, P=0.271 and $F_{6,53}$=0.748, P=0.614). However, there was a significant main effect of treatment ($F_{4,53}$=5.371, P<0.001). The number of Paneth cells fell from 1.3 ± 0.4 cells/crypt on day 73 to 1.1 ± 0.4 cells/crypt on day 94 in the primary-not challenged hamsters. Naïve-challenged hamsters also showed a decline in Paneth cells from 2.1 ± 0.3 cells/crypt on day 73 to 1.6 ± 0.5 cells/crypt on day 94. However, the number of Paneth cells in both abbreviated primary-challenged and primary-superimposed challenged groups fluctuated during the time of this experiment indicating that time was not

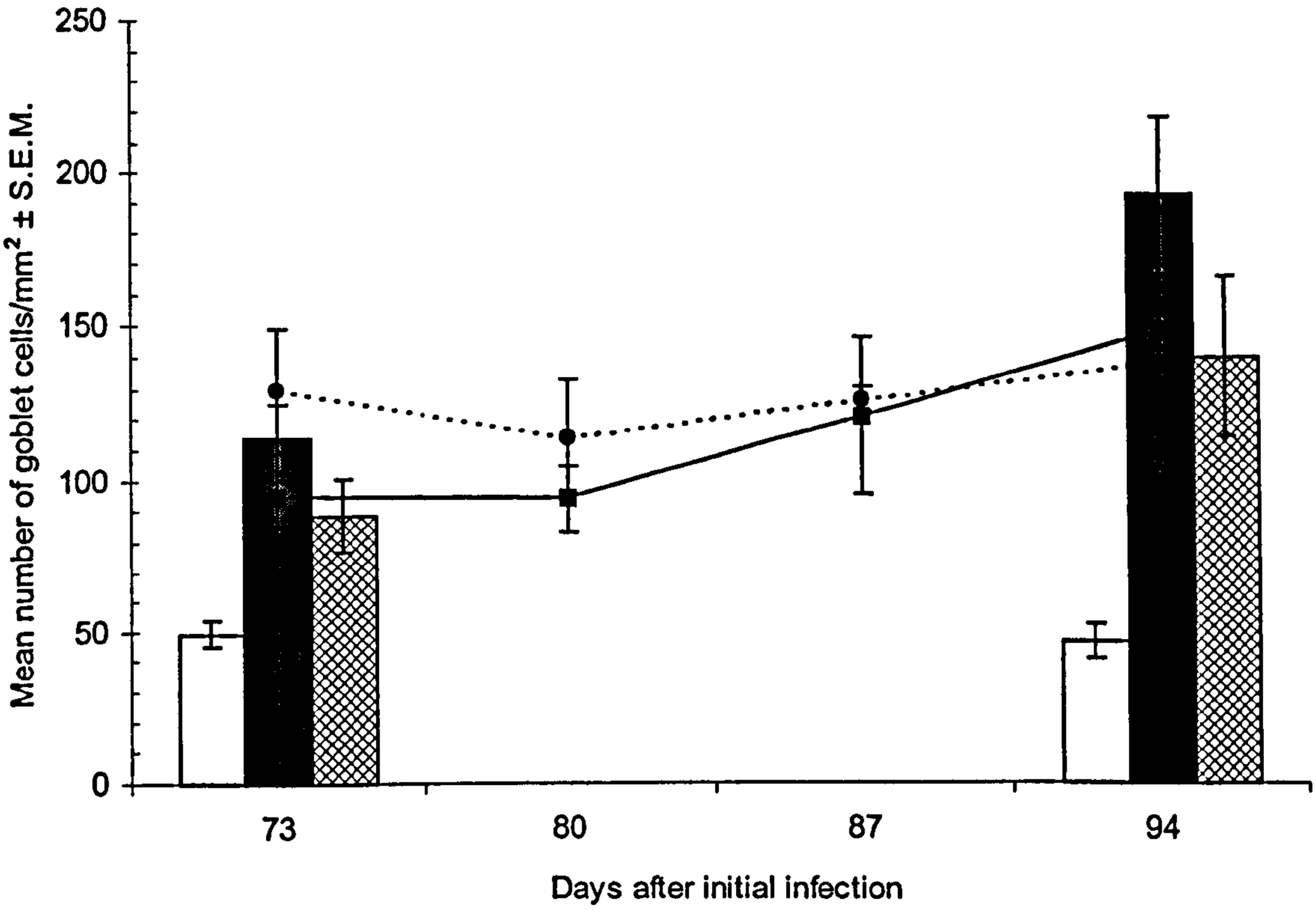


Figure 4.12 – Mean number of goblet cells/mm² (± S.E.M.) in the intestinal tissue of:

Naïve-uninfected hamsters (▭).

Primary infected hamsters with *A. ceylanicum* (▬).

Naïve-challenged hamsters (▨).

Abbreviated primary-challenged hamsters (—■—).

Primary-superimposed hamsters (·····●·····).

<u>Statistical analysis:</u>

Analysis of these data by 2-way ANOVA with treatment (5 level, naïve-not challenged, primary-not challenged, abbreviated primary-challenged, naïve-challenge and primary-superimposed challenged) and time (4 level, 73, 80, 87 and 94 days after initial infection on day 0) revealed:

Main effect of treatment ($F_{4,53}$=8.093, P<0.001)

Main effect of time ($F_{3,53}$=3.642, P<0.05)

Interaction between time and treatment ($F_{6,53}$=0.944, P=0.472)

Model R^2=0.350

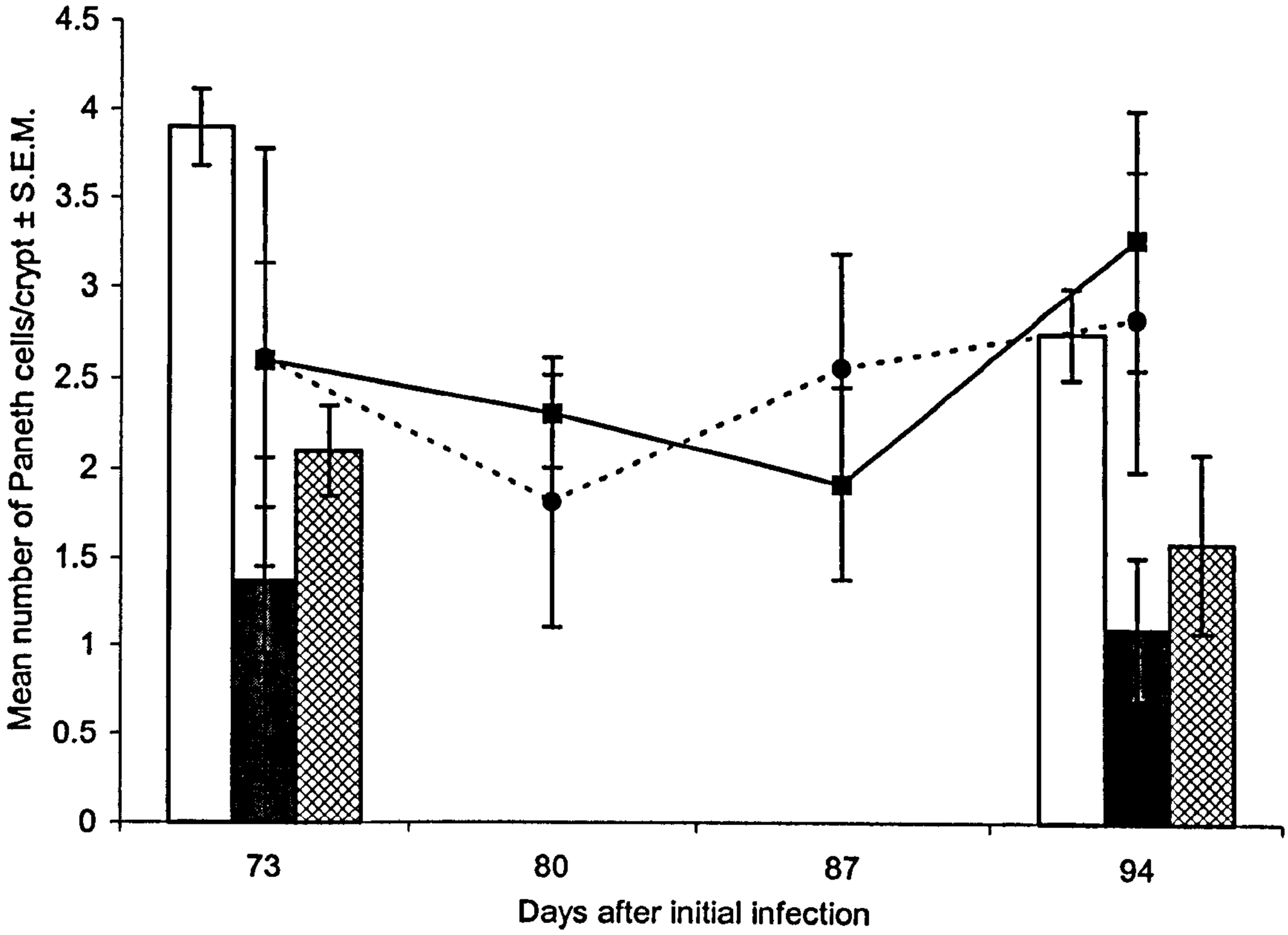


Figure 4.13 – Mean number of Paneth cells/crypt (± S.E.M.) in intestinal tissue of:

Naïve-uninfected hamsters (▭).

Primary infected hamsters with *A. ceylanicum* (▬).

Naïve-challenged hamsters (▨).

Abbreviated primary-challenged hamsters (—■—).

Primary-superimposed hamsters (·····●·····).

<u>Statistical analysis:</u>

Analysis of these data by 2-way ANOVA with treatment (5 level, naïve-not challenged, primary- not challenged, abbreviated primary-challenged, naïve-challenged and primary-superimposed challenged) and time (4 level, 73, 80, 87 and 94 days after initial infection on day 0) revealed:

Main effect of treatment ($F_{4,53}$=5.371, P=0.001)

Main effect of time ($F_{3,53}$=1.339, P=0.271)

Interaction between time and treatment ($F_{6,53}$ =0.748, P=0.614)

Model R^2=0.179

significant here, (r_s=0.035, n=20, P=0.884 and r_s=0.052, n=17, P=0.843, respectively).

4.3.2.7 – Eosinophil responses

Figure 4.14 shows changes to eosinophil numbers in the mucosa of hamsters' intestines infected with *A. ceylanicum* and the statistical 2-way ANOVA analysis is given in the legend. There was a highly significant main-effect of treatment ($F_{4,55}$=18.029, P<0.001). Moreover, significant interaction between treatment and time was observed ($F_{6,55}$=3.416, P<0.01). However, there was no significant main effect of time ($F_{3,55}$=2.181, P=0.101). It is clear from the figure that all infected groups had a markedly elevated number of eosinophils compared to the naïve-control hamsters at all the times they were examined but, nevertheless, these groups showed no marked changes over time. The correlational test showed that the reduction in abbreviated primary-challenged groups was significant over the period of the experiment (r_s=-0.566, n=20, P<0.01). However, primary-superimposed challenged groups showed no significant differences with time (r_s=-0.164, n=19, P=0.503), which could be related to the low value on day 80 before the increase towards the end of this experiment.

4.4 – DISCUSSION

Experiments with hookworm infections in dogs (Carroll and Grove, 1985a; Carroll and Grove, 1985b) and hamsters (Behnke *et al.*, 1997; Gupta and Katiyar, 1985; Menon and Bhopale, 1985b) showed that single pulse primary-infections induced significant protection against secondary exposure. The demonstration of acquired resistance to *A. ceylanicum* in hamsters was important because the hamster-*A.ceylanicum* combination represents a rodent model in which mature hosts challenged by hookworm larvae achieve patent infections. This model may be investigated to achieve a detailed analysis of the

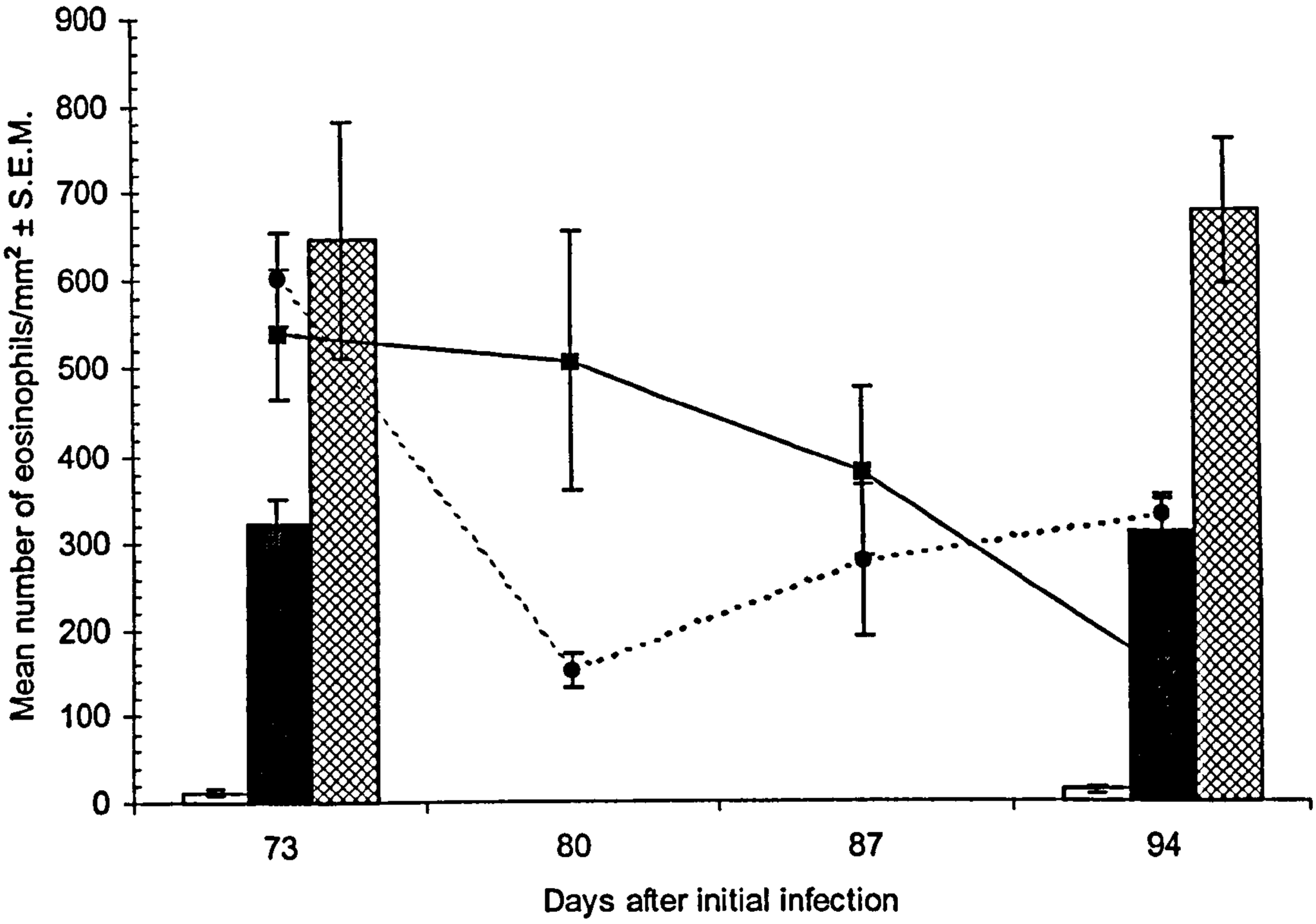


Figure 4.14 – Mean number of eosinophils /mm² (± S.E.M.) of intestinal tissue of:

Naïve-uninfected hamsters (▭).

Primary infected hamsters with *A. ceylanicum* (▬).

Naïve-challenged hamsters (▨).

Abbreviated primary-challenged hamsters (—■—).

Primary-superimposed hamsters (····●····).

<u>Statistical analysis:</u>

Analysis of these data by 2-way ANOVA with treatment (5 level, naïve-not challenged, primary-not challenged, abbreviated primary-challenged, naïve-challenged and primary-superimposed challenge) and time (4 level, 73, 80, 87 and 94 days after initial infection on day 0) revealed:

Main effect of treatment ($F_{4,55}$=18.029, P<0.001)

Main effect of time ($F_{3,56}$=2.181, P=0.101)

Interaction between time and treatment ($F_{6,56}$=3.416, P<0.05)

Model R^2=0.555

components necessary for the expression of protective immunity to hookworms in hosts. Analysis of such work was initiated by Garside *et al.*, (1990) and developed further by Behnke, *et al.*, (1997) and we continued and extended such analysis in the experiments reported in this chapter.

The effect observed on host parameters, such as intestinal architecture, mast and goblet cell infiltration, changes in Paneth cells and eosinophils during primary infection were as expected from previous studies and as reported in previous chapters. The understanding of how the immune parameters respond to infection as presented in earlier experiments by Garside, *et al.* (1989) was extended. The severity of pathology, reflected by declining villous height and increasing crypt depth, and increases in mast cells, goblet cells and eosinophils varied according to the combination of primary infection and challenge utilized in the present experiment.

4.4.1 – Architectural changes in the intestinal mucosa

It is well known that the small intestine is critically important to the host as the principal site for the absorption of most nutrients produced by digestion of the food into the blood (Campbell *et al.*, 1999; Kamal, 2001). However, the internal surface and its structure may alter due to a variety of factors. As was noted in other chapters of this thesis and some earlier studies, intestinal tissue has a fast turnover rate, lesions being repaired relatively rapidly within two weeks of treatment (Chapter 3, Experiment 7).

Both experiments presented in this chapter showed the usual range of villous height and crypt depth in naïve hamsters, from 753-812 µm in the first experiment (Figure 4.2) and 954-955 µm in the latter (Figure 4.9). Again, the average villous height was found to be close to the values from experiments reported earlier in chapter 3 (Figure 3.10). It was also found that the height of villi in primary hamsters was reduced as low as 57 to 64 µm. This was found to be a continuation of the reduction in villous height that commences when worms mature in the intestine. Villi returned to their normal height following anthelmintic treatment, which again confirms the findings in Experiment 7 (presented in

Chapter 3) where the levels begun to return to normal only two weeks after Ivermectin treatment.

It is obvious from the results illustrated in figure 4.2 that, after initial infection, villous height in the abbreviated primary-challenged groups declined continuously over time from the normal level to less than 50% of its height. This suggests that villous height again declined from the height restored after the anthelmintic treatment. Naïve-challenged hamsters had reduced villous height on day 73 relative to naïve animals, indicating as in earlier experiment, a rapid response taking place within 10 days of challenge but perhaps surprisingly villous height did not continue to decline over the following 21 days and was much the same on day 94. However, worm burdens were lower on day 94 (i.e. 31 days after infection) than on day 73 and in earlier experiments and this may simply be a dose effect.

It was also found that a concurrent steady increase in crypt depth was significant with regards to time after initial infection in both abbreviated primary-challenged animals from Experiment 1 and 2. The changes in crypt depth of naive-challenged hamsters between days 73 and 94, which were not associated with similar changes in villous height, suggest that worm feeding activities are not the only factor affecting mucosal architecture. Thus, another mediator possibly directed by Th2 cells may be involved in bringing about these changes.

In the first experiment presented in this chapter, worm burden showed an expected reduction over time in the primary groups of hamsters as well as naive-challenged animals after the initial infection. In the second experiment of this chapter, however, an increase in worm burden was noticed in the primary challenged group. It is possible that this arose as an experimental error through a higher than intended dose of L3 being administered to the group killed on day 94. Alternatively, it may have arisen through natural variations in establishment.

4.4.2 – Mast cells

It is confirmed in this chapter that MMC in the intestine of naïve hamsters did not exceed a mean number of 13 cells/mm² in the intestinal tissue (Figure 4.4 and 4.11) (also see Chapter 3). The total number of mast cells was considerably higher in primary hamsters in both experiments with a slight reduction between days 73 and 94 in the former and very little increase between days 73 and 94 in the latter, which could be related to the mean number of worms present. However, when hamsters were exposed to infection on day 63, the total number of mast cells elevated dramatically from the normal level of around 10 cells/mm² to 33.688 cells/mm² on day 94 (31 days after infection) of Experiment 1, and to 35.020 cells/mm² on the same day of Experiment 2.

As presented in the Figure 4.4, it was found that mast cells increased slowly with time after the abbreviation of the initial infection followed by a challenged infection with 50 L3 *A. ceylanicum*, which suggests that components of the immune system have acted in the intestine and prevented the new infection from being fully established. Despite the dip in worm burden recorded on day 87 in the first experiment, the number of mast cells continued to increase steadily with time. On the other hand, in the second experiment mast cells showed a decrease from the first day of autopsy to day 87 and then an increase towards the end. This different pattern of response in Experiment 2 was found to contrast with that in Experiment 1 and it may be relevant that the mean number of worms established after the challenge infection was lower in Experiment 2.

Mast cell numbers in primary-superimposed infected animals were at their highest level on day 73 compared with other treatments then continuously declined until day 87 then increased slightly towards the end of the experiment. This could be due to the high mean number of adult worms recovered on that day, which also show signs of a reduction afterwards.

4.4.3 – Goblet cells

As in all the other experiments presented in the previous chapters, goblet cell numbers in naïve-not challenged hamsters were found to be very similar at the different times examined, with low values recorded in both Experiments. The number never exceeded 60 cells/mm^2 at any time during the course of this experiment or others as reported earlier.

Similarly, the responses to primary infection were dramatically higher than those of naïve animals with a significant increase between days 73 and 94. However, data from both experiments showed different responses in the total number of goblet cells in abbreviated-challenged hamsters. Goblet cell numbers increased until a plateau was achieved towards the end of the first experiment whilst steadily increasing in Experiment 2. Interestingly, the goblet cell responses in primary-superimposed challenge animals was seen to be similar to that of the abbreviated primary-challenged groups, although there was a slight difference between the two treatments on day 73 when goblet cell numbers in the abbreviated primary-challenged were below the 100 cells/mm^2 while they reached 150 cells/mm^2 in the primary-superimposed challenge groups.

4.4.4 – Paneth cells

Sections of small intestine from hamsters undergoing the range of treatments, stained with phloxin-Tartrazine showed differences in Paneth cell numbers. Naïve normal tissue showed higher numbers of Paneth cells with wide variation within groups. This number fell in hookworm infected animals.

In contrast to Paneth cell types in other rodents, Paneth cell numbers here, in hamsters, were found to be reduced when hamsters became infected. Once this infection was removed using Ivermectin, there was a sharp increase to a level higher than that found in naïve-not challenged groups. It is also clear from the figures (Figure 4.13) that marked differences can be seen between the

response in abbreviated primary-challenged animals and primary-superimposed challenge infection groups.

In the experiments described in this chapter, the usual number of Paneth cells in normal uninfected hamsters (naïve) ranged between 2-5 cells/crypt in both experiments. It was also found that hamsters with a primary infection of *A. ceylanicum* (primary) showed a decline in Paneth cells during the course of infection which resembled the responses seen in the case of diseases such as Crohn's, ulcerative colitis and tumours (Lewin, 1969c; Stuart and Gent, 1998). A significant reduction in the percentage of crypts containing Paneth cells was found in rats given cadmium as the chloride salt in the drinking water (Phillpotts, 1986). The reduction in Paneth cells during these sorts of diseases was possibly related to non-specific injury which may be due to the disease process, which might also be the case in this experiment, i.e., the feeding activities of A. *ceylanicum* during their browsing on the surface of the mucosa. However, this may only be one of a number of more complicated factors that could be involved. It is clear from the results in Figures 4.6 and 4.13 that Paneth cells showed a profound depression by approximately 50% in the primary groups compared to those observed in naïve hamsters. This result confirmed previous results reported in earlier chapter (Chapter 3) and indicated that as long as the worms were present in the intestine, Paneth cells responded by a reduction. It also appears that these changes might be time-dependant as this reduction in Paneth cell numbers continued or was sustained over the course of both experiments in this groups (primary), despite the already low values on day 73. On the other hand, Paneth cell numbers were higher in the abbreviated primary-challenged groups in Experiment 1 compared to other groups, except that seen in the abbreviated primary group, which showed high numbers of Paneth cells, exceeding 4 cells/crypt on both days (i.e. 73 and 94). This finding was different in Experiment 2 when the numbers of Paneth cells in naïve groups were higher. It is suggested that naïve groups in this case were cleaner and therefore Paneth cells were higher than in naïve-not challenged animals from the first experiment. Similar Paneth cell responses were also found in the primary-superimposed challenge infection in Experiment 2 (Figure 4.13). However, the differences in worm burdens were marked between the two groups (abbreviated primary-

challenged and primary-superimposed challenge). Nevertheless, the mean worm burdens in the later group consisted of large adult worms as well as L4 stages and some pre-adult worms. Therefore, one suggestion for this is that the adult worms in the intestine of hamsters from the primary-superimposed challenge group in Experiment 2 are in fact the survivors from the primary infection.

4.4.5 – Eosinophils

The lowest levels of eosinophils were recorded in naïve hamsters during the course of both Experiments (1 and 2) with a marked increase during the primary infection. Changes in eosinophils during challenge and superimposed infections with *A. ceylanicum* were reported here for the first time and thus, it is difficult to compare the results with any previous data. The mean number of eosinophils did not exceed 40 cells/mm^2 in naïve-not challenged groups of hamsters, which confirms that eosinophils at these densities exist in the intestinal mucosa. Moreover, the number of eosinophils increased dramatically when the animals were given the hookworm infection, possibly elicited in part by the pathology at the site of attachment (Behm and Ovington, 2000).

In the two experiments reported in this chapter worm burdens were variable but in the abbreviated primary-challenged animals they were particularly low and constant with perhaps just a suggestion of some loss towards day 94. The difference in worm burden between these groups and the naïve-challenged animals shows that in both experiments, a considerable proportion of the challenge infection failed to survive until day 73 (i.e. 10 days after challenge). In both experiment abbreviated primary-challenged groups had elevated eosinophil number, but whereas these remained high in Experiment 1, they fell between days 73 and 94 in Experiment 2. Interestingly, although primary-superimposed challenge hamsters had higher worm burden than abbreviated primary-challenged animals in Experiment 2, the eosinophil numbers were lower in the former on days 80 and 87 and higher on day 94.

In abbreviated primary-challenged animals and in primary-superimposed challenge groups most of the challenge infection was lost within 10 days of challenge in agreement with Behnke *et al.*, (1997). However, there were clearly marked responses at this time as well as persistent responses that lasted until 31 days after infection. Despite this, some adult worms persisted throughout, although evidence for some loss of adult worms was obtained. It is therefore suggested that larval *A. ceylanicum* are susceptible to the rapid inflammatory responses that are initiated in the intestinal mucosa. However, some always manage to establish and develop to adult stages, which are considerably more difficult for the host to eliminate. It is the prolonged and sustained response lasting for 31 days after challenge that in some experiments manages to lower worm burdens eventually, but not always. In these experiments, some adult worms always persisted until day 94 (i.e. 31 days after challenge) irrespective of the intensity of the intestinal inflammatory responses.

Finally, the experiments reported in this chapter have established that challenge infection with *A. ceylanicum* is resisted by hamsters whether the primary infection persists or not. However, resistance does seem to be more marked in the absence of persisting adult worms, adding weight to data suggesting that adult hookworm are immunomodulatory (Loukas and Prociv, 2001).

CHAPTER FIVE

ASSOCIATED CELLULAR AND MUCOSAL CHANGES DURING TRICKLE INFECTION WITH *ANCYLOSTOMA CEYLANICUM* IN HAMSTERS.

5.1- SUMMARY

An experiment was carried out to assess the consequences of a trickle infection with *A. ceylanicum* in hamsters. Changes in the intestinal architecture and in infiltration by inflammatory cells, such as mast cells, goblet cells, Paneth cells and eosinophils were examined in relation to changes in hookworm burden in hamsters.

As in the single pulse primary infection, hamsters exposed to trickle infection responded with major changes in the intestinal architecture and in inflammatory cells. However, there were distinct differences in the kinetics of response to these two types (primary and trickle) of infection. It was found that the time dependant reduction in villous height and the increase in crypt depth in animals exposed to trickle infection were both initially slower but eventually equalled and exceeded, respectively, the response in hamsters given primary infection. Similarly, changes in the mitotic figures of epithelial cells in the mucosa and the mast cell response were both initially slower and less intense in trickle infected hamsters, but eventually exceeded the response to primary infection. Furthermore, the eosinophil response was found to be initially greater in trickle infection and overall more persistent. In contrast, both goblet cell and Paneth cell responses were less marked in trickle infected animals compared to primary infected groups. These results were discussed in the context of host protective resistance to infection with *A. ceylanicum*.

5.2 – INTRODUCTION

Humans and domestic animals are continuously exposed to varying quantities of infective larvae in nature. Nevertheless, the immunology of repeatedly administrated antigens is a poorly developed field (Andrew *et al.*, 1984; Saklayen *et al.*, 1984) that warrants further investigation. Saklayen *et al.* (1984) stated that the repeated administration of certain antigens, such as hen egg albumin (OVA), can induce antigen-specefic tolerance. The IgE response in

this study was most effectively suppressed by antigen feeding prior to or after parenteral immunization. The effect on the IgG response paralleled that observed on the IgE response, but the magnitude of suppression was less pronounced. The IgA response was enhanced rather than suppressed if the interval between feeding and parenteral immunization was short or if the animals were antigen-fed after the intraperitoneal priming. Such studies help to explain the existence of tolerance to otherwise antigenic foreign molecules in food and also provide an avenue for understanding the best response to repeatedly acquired infectious organisms, some of which may cause chronic infections.

Jenkins and Phillipson (1971) found that when rats were exposed to *N. brasiliensis* at a dose of five larvae per week day (an attempt to mimic natural infections in nature) worm burdens accumulated, resulting in parasite survival for more than 12 weeks, which is considerably longer than normal. However, trickle infections were only successful when initiated in young rats; older animals developed resistance quickly so neonatal unresponsiveness may be a contributing factors (Jenkins, 1974).

Stability in *T. muris* worm burdens following trickle exposure was not found in mouse strains, such as NIH and CFLP unless they were concurrently infected with *H. polygyrus* or were immunocompromised (Behnke *et al.*, 1984; Behnke and Wakelin, 1973). In control CFLP mice trickle infections did not survive to maturity but in the slower responder $C57BL_{10}$ mice, egg production began on day 34 and continued until day 90 with some mature worms present at autopsy (Behnke *et al.*, 1984). It was suggested by Behnke *et al.* (1984) that this strain of mouse may have high immunological thresholds. They suggested that the larvae from the initial infection had an opportunity to complete their development to the adult stage before the immune response began to operate against subsequent doses of incoming infective stages and therefore were able to survive to autopsy on day 90. More recent study on *T. muris* in mice by Bancroft, *et al*, (2001) showed that it is possible to build up the level of infection to a threshold level beyond a level that would normally trigger the type 2 response and expulsion and this threshold level was dependant upon host genetic background, supporting the previous finding.

Intestinal nematode infections constitute an important problem in the livestock agricultural industry and are responsible for severe economic losses (Michel, 1976; Michel, 1982; Michel, 1985). In some studies, high contamination of the pasture with infective larvae was found to be seasonal; exceeding 900 L3/kg in mid summer (Thomas, 1974). When cattle were experimentally exposed to repeated doses of *O. ostertagi* a continuous turnover in worm burden was detected, with new worms being acquired as the old ones were lost. Therefore, the life span of adult worms was reduced from a potential value of about 100 days in a single pulse infection (Michel *et al.*, 1978) to an average of about 26 days under field conditions (Michel, 1970). It is the brevity of the lifespan which is the essential factor in regulating populations (Michel, 1982). Thus, adult worms are continuously lost and replaced by incoming infective larvae. Resistance to infection increases with continuous exposure, so that gradually incoming larvae are less successful and replacement rates fall, explaining the eventual decline in worm numbers (Donald and Waller, 1982; Michel, 1970). Despite the higher worm burden of *O. ostertagi* under repeated infection, similar kinetics were observed in humans with *Ascaris lumbricoides* infection. Elkins *et al.* (1986) calculated that the longevity of *A. lumbricoides* may be only 12 months, despite an extremely high prevalence across all age groups in the communities studied. Larval and adult parasites were found throughout the different age groups, suggesting that as in *O. ostertagi* a continues turnover of the worm population was taking place.

On the other hand, animals repeatedly infected with *T. vitrinus* or *T. colubriformis* show a different pattern of events; the worms are not regulated by a turnover of the adult population (Donald and Waller, 1982). In contrast to *O. ostertagi*, when sheep are exposed to daily infection with *Trichostrongylus*, the adult worm burdens accumulate until resistance develops to incoming larvae. Even when larval acquisition has been completely terminated, adult worms persist for varying periods of time before they also are expelled (Donald and Waller, 1982; Jackson *et al.*, 1983). More complex regulation of the *H. contortus* populations in sheep grazing on endemic pastures was found by Barger *et al.* (1985) and Courtney *et al.* (1983). The former found that sheep accumulated worm burdens for the first 4 weeks reaching a plateau following repeated

inoculation with 2400-4800 larvae/week, after which establishment of further incoming larvae was prevented. Expulsion of adult worms followed in weeks 12-14. In contrast, sheep given 600-1200 larvae/week did not expel the accumulated adult worms within the 15 week experimental period. Therefore, these experiments are not compatible with worm turnover of the kind seen in *O. ostertagi.* However, this suggests development of resistance to infection, reflected in reduced establishment, increasing arrest among incoming larvae and the loss of establishment adults, triggered by the density of the worm population and the age of the host. Moreover, immunomodulatory activities by the adult worms may enable the survival of the adult worms while anti-larval immunity develops in the host. Eventually, it is possible that parasite immunomodulatory factors are also neutralized which enables the expulsion of adult worms to take place (Behnke, 1987b).

There has been little work carried out on repeated infection with hookworms in dogs. Experiments carried out by Herrick (1928) revealed that there was no increased resistance to any subsequent infection of hookworm larvae when worms were removed using anthelmintic treatment following initial infection. However, some evidence of resistance by dogs to infection was seen when challenge infection with *A. caninum* followed the initial infection. Another attempt was made by McCoy (1931) who repeatedly infected dogs with *A. caninum* and found that parasite numbers were regulated under the trickle-infection regimes. More recent work involving trickle infection with *A.ceylanicum* was carried out in hamsters and it was found that worm burdens were regulated at very low levels throughout the course of several experiments (Brailsford and Behnke, 1992a). Doses of 5-30 *A. ceylanicum* larvae were administered to inbred DSN hamsters on a weekly basis and then the animals were killed at intervals for estimation of established worms. The results of these experiments were not entirely compatible with worm turnover but rather suggested that resistance was elicited by the worms which established initially from the first few inocula. The development of resistance was reflected in the reduced establishment and increasing arrest among larvae from subsequent inocula.

In summary, the literature reviewed above indicates that several different mechanisms may operate to mediate host resistance in animals exposed to

trickle infection. Overall, this is still a poorly understood process, and particularly in the context of hookworm infections. The associated changes in the gut mucosa have not been studied. To remedy this gap in knowledge a quantitative investigation was conducted focussing on the changes occurring in DSN hamsters exposed to repeated infection with *A. ceylanicum* over 70 days.

5.3 – EXPERIMENTAL DESIGN AND RESULTS

An experiment was carried out to observe the effects of a trickle infection with *A.ceylanicum* on the intestinal cellular immune response in hamsters. This infection protocol attempted to mimic natural acquisition of larvae under field condition when man and animals are continually exposed to varying quantities of infective larvae of their respective parasites (e.g. hookworm in man and *Ostertagia* in cattle).

5.3.1- Trickle infection with *A. ceylanicum*

Sixty-five adult female DSN hamsters were employed for this experiment. The experimental design is shown in Table 5.1. Fifteen hamsters were divided into three groups of five in each cage; they were left uninfected and killed on days 14, 28 and 56-post infection. Another fifteen hamsters received a primary infection with 50 L3 *A.ceylanicum* and were killed on the same days as the uninfected groups. Twenty-five hamsters received a weekly infection with 30 L3 *A.ceylanicum* and were killed in groups of five on days 14, 28, 42, 56 and 70. Finally, nine groups of two hamsters in a cage received only the primary infection with 30 L3 *A.ceylanicum* to test the infectivity of the larvae used at each infection. These 'infectivity' controls were set up at regular weekly points throughout the experiment and killed two weeks after infection, with worms being counted at autopsy 14 days post-infection.

Table 5.1 – Experimental design and worm recovery in an experiment to assess the effect of trickle infection with A. ceylanicum.

Treatments	No. of hamsters	Dose of L3 given	Mean no. of worms recovered	Day killed
1	5	Nil	Nil	14
	5	Nil	Nil	28
	5	Nil	Nil	56
2	5	30 L3 Weekly	6 ± 1.789	14
	5	30 L3 Weekly	5.2 ± 1.8	28
	5	30 L3 Weekly	2.8 ± 1.2	42
	5	30 L3 Weekly	4.8 ± 0.735	56
	5	30 L3 Weekly	2.6 ± 0.510	70
3	5	50 L3	11.8 ± 3.089	14
	5	50 L3	13.6 ± 3.140	28
	5	50 L3	8.6 ± 2.6	56
4- Tracers	18	30 L3	See Figure 5.2	Weekly

Treatments code:

1- Received no infection and killed at different days
2- Infected weekly with 30 L3 *A. ceylanicum* until the day of autopsy (Trickle infection).
3- Infected with 50 L3 of *A. ceylanicum* on day 0 (Primary infection).
4- Tracer hamsters were infected with 30 L3 *A. ceylanicum* and killed in groups of two, weekly after 14 days post infection to check larval infectivity.

5.3.1.1 – Total worm recovery

The total number of worms recovered from primary and trickle infected hamsters are illustrated in Figure 5.1. (B) with different development stages of *A. ceylanicum* detailed in Figure 5.1. (A). As can be seen from the figure, the worm burdens in primary and trickle infections varied between the groups. Two-way ANOVA revealed that there was a significant main effect of treatment and time on worm burdens ($F_{1,32}$=14.025, P<0.01 and $F_{4,32}$=3.392, P<0.05, respectively) but no interaction between treatment and time ($F_{2,32}$=0.465, P=0.633). Changes in worm burden with time from each treatment were also analysed by Spearman's rank test. In hamsters given just the single primary infection, worm burdens appeared to decline with time, but this reduction was not significant, although the probability was just outside the cut off for significance (r_s=-0.493, n=15, P=0.062). In contrast, in trickle infected hamsters, worm burden fell significantly with time (r_s=-0.442, n=25, P<0.05).

Figure 5.2 shows the mean number of worms recovered from groups of hamsters two weeks after infection with each of the separate doses, employed for the trickle infection. The mean numbers of worms recovered fluctuated but overall were much as expected with approximate establishment ranging from 13 to 33 %.

5.3.1.2 – Assessment of intestinal pathology

Changes in the intestinal architecture of the hamsters are shown in Figure 5.3 Both primary and trickle infections elicited marked changes to the intestinal mucosa compared to naïve, uninfected hamsters. The villous heights in the trickle-infected hamsters declined with time from a level between 1044-1091µm in naïve, uninfected groups to a level of 155 ± 17.757 on day 56 after infection with a slight increase to 240 µm ± 48.477 on day 70. A similar decrease in the height of villi was observed in the primary infection from day 14 to 56 with more profound changes than those noticed in the trickle infected animals. This is

Figure 5.1 – Mean number of worms recovered from the intestine of primary and trickle infected hamsters with *A. ceylanicum*.

A- Total number of worms during a primary infection and numbers of L4 and adult worms during trickle infection

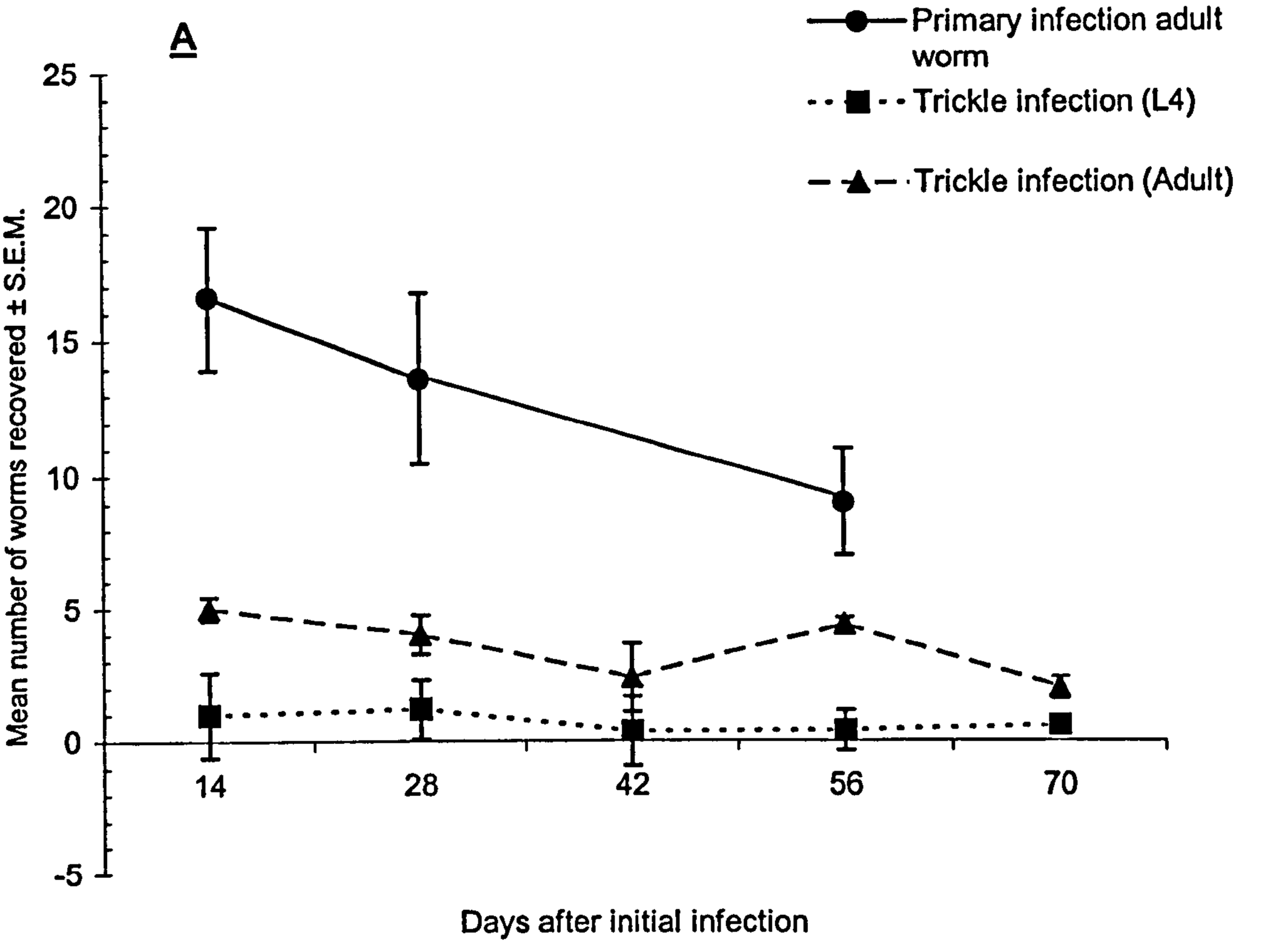
B- Total No. of worms in primary and trickle infected hamsters.

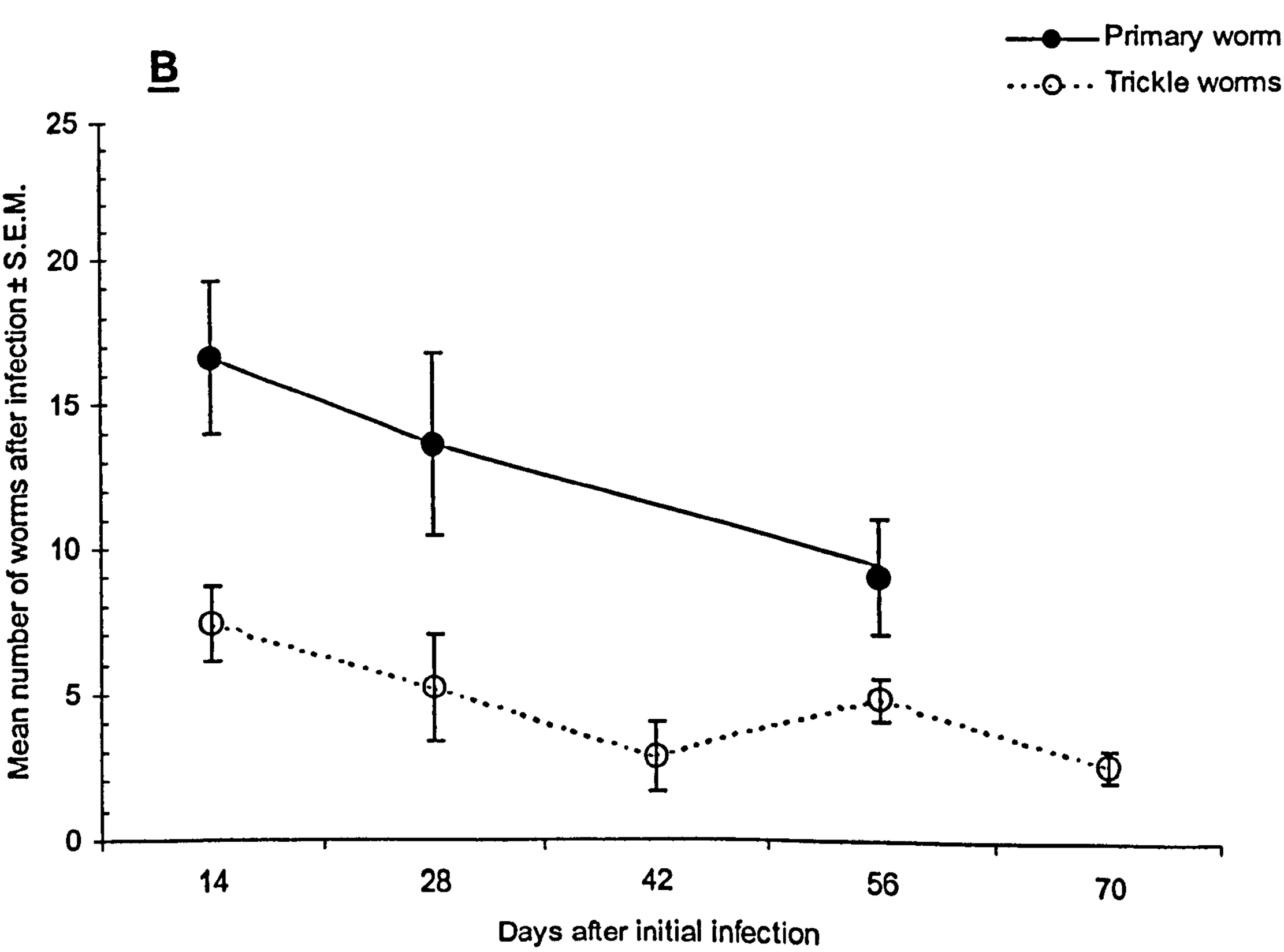
Statistical analysis

Analysis of worm burden [Log (X+1)] transformated data by 2-way ANOVA with treatment (2 levels, primary and trickle) and time (5 levels, 14, 28, 42, 56 and 70) revealed:

Main effect of time	$F_{1,32}=3.392$, $P<0.05$
Main effect of treatment	$F_{4,32}=14.025$, $P<0.01$
Interactions between treatment and time	$F_{2,32}=0.465$, $P=0.633$

Model $R^2=0.594$


A
Primary infection adult worm
Trickle infection (L4)
Trickle infection (Adult)
Mean number of worms recovered ± S.E.M.
25
20
15
10
5
0
-5
14
28
42
56
70
Days after initial infection


B
Primary worm
Trickle worms
Mean number of worms after infection ± S.E.M.
25
20
15
10
5
0
14
28
42
56
70
Days after initial infection

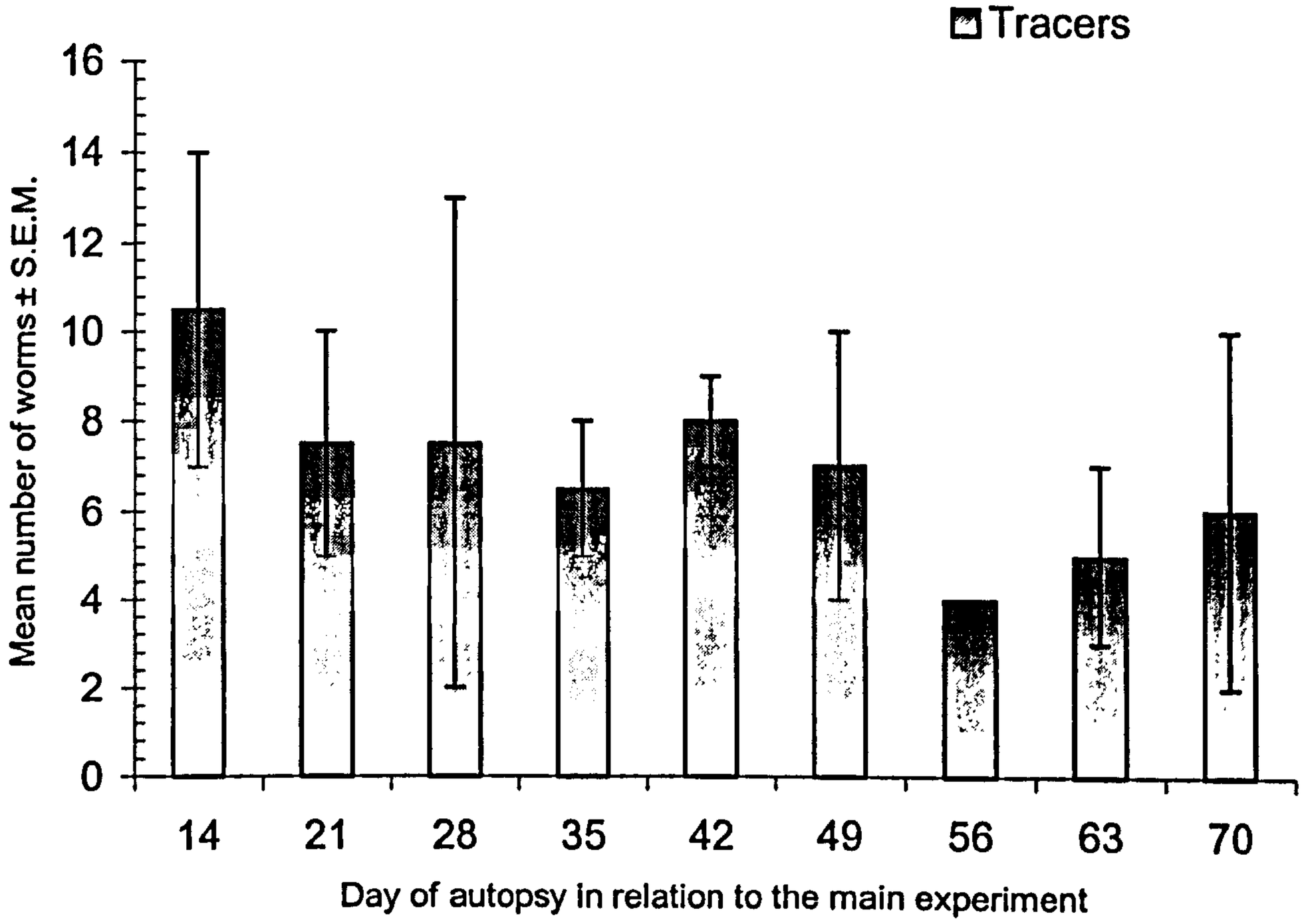


Figure 5.2 – Mean number of worms recovered from tracer hamsters after two weeks of infection. Each group received 30 L3 *A. ceylanicum* two weeks before the day of autopsy shown on the X axis.

Figure 5.3 – Measurements of villous height (+) and crypt depth (-) (±S.E.M.) in the intestine of:

Naïve-uninfected hamsters (▭).

Primary infected hamsters with *A. ceylanicum* (▨).

Trickle infected hamsters with *A. ceylanicum* (–▲–).

Statistical analysis of changes in villous height

Analysis of villous height by 2-way ANOVA with treatment (2 levels, primary and trickle) and time (5 levels, 14, 28, 42, 56 and 70) revealed:

Main effect of time	$F_{4,44}$=92.628, P<0.001
Main effect of treatment	$F_{2,44}$=643.865, P<0.001
Interactions between treatment and time	$F_{4,44}$=35.565, P<0.001

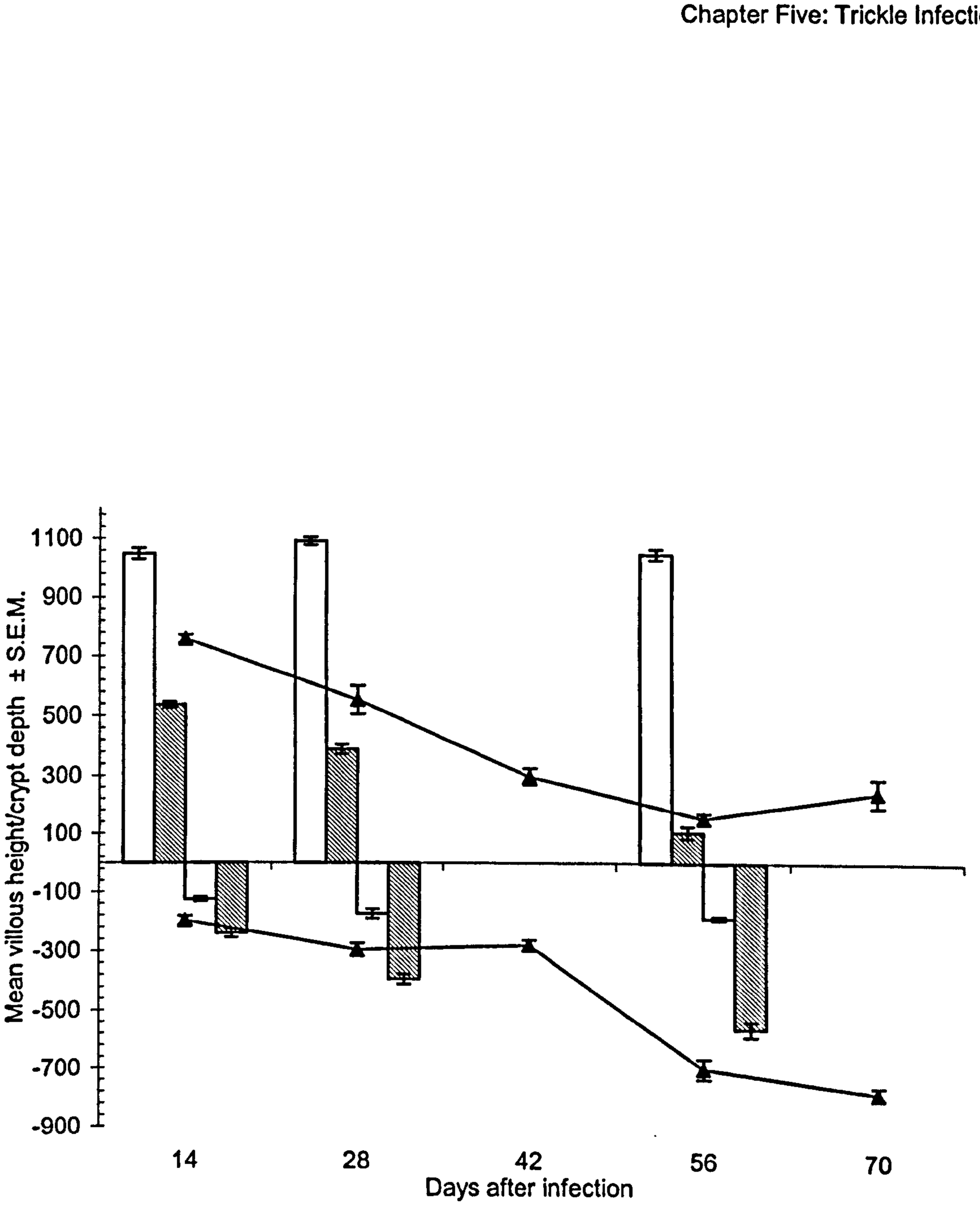
Model R^2 = 0.974

Statistical analysis of changes in crypt depth

Analysis of crypt depth by 2-way ANOVA with treatment (2 levels, primary and trickle) and time (5 levels, 14, 28, 42, 56 and 70) revealed:

Main effect of time	$F_{4,44}$=183.256, P<0.001
Main effect of treatment	$F_{2,44}$=146.307, P<0.001
Interactions between treatment and time	$F_{4,44}$=39.573, P<0.001

Model R^2 = 0.960


1100
900
700
500
300
100
-100
-300
-500
-700
-900
Mean villous height/crypt depth ± S.E.M.
14
28
42
56
70
Days after infection

possibly related to the heavier worm burdens resident in hamsters with the single dose primary infection. Two-way ANOVA was used to analyse the effect of time and treatment on villous height and the results showed that there was a highly significant effect of both time and treatment on the length of the villi ($F_{4,44}$=92.628, P<0.001 and $F_{2,44}$=643.865, P<0.001, respectively). There was also a highly significant interactions between time and treatment on villi ($F_{4.44}$=35.565, P<0.001). Furthermore, there was significant villous atrophy with time in both primary infected (r_s=-0.945, n=15, p<0.001) and trickle infected groups of hamsters (r_s=-0.839, n=25, p<0.001), but no changes in the naïve groups (r_s=-0.94, n=15, p=0.738).

There were similar marked changes in the depth of crypts in the intestine between naïve-uninfected groups, primary infected and trickle infected hamsters with time and treatment. Crypt depth increased with time from a range of 126-185 µm in naïve animals to 780 µm ± 21.5 on day 70 in the trickle infection (r_s=0.902, n=25, p<0.001) with partial return from 296.250 µm ± 21.254 on day 28 to 280.5 µm ± 18.408 on day 42, which may be associated with the change in worm burden in the intestine. Similar differences in the depth of the crypt with time were also observed throughout the experiment in primary-infected hamsters (r_s=0.945, n=15, p<0.001). Although there were also significant changes with time to the depth of the crypt in the uninfected control groups (r_s=0.757, n=15, P<0.001) the relative change was very small, ranging from a mean of 126.250 µm ± 7.624 on day 14 to 174.500µm ± 15.307 on day 28 and 185.250 µm ± 7.790 on day 56. 2-way ANOVA with treatment (3 levels) and time (5 levels) revealed a highly significant main effect of time ($F_{4,44}$=183.256, P<0.001) and treatment ($F_{2,44}$=146.307, P<0.001). Moreover, the interaction between treatment and time was also highly significant ($F_{4,44}$=39.573, P<0.001).

5.3.1.3 – Cellular division in the crypt of Lieberkuhn

Changes in the numbers of mitotic figures in the Crypts of Lieberkuhn are illustrated in Figure 5.4 and the statistical analysis is given in the legend. As can

be seen both primary and trickle infection had a marked and significant time dependant effect on mitotic numbers and they were significantly higher than in naïve animals (the main effect of time and treatment, $F_{4,44}$=58.408, P<0.001 and $F_{2,44}$=137.381, P<0.001, respectively). Interaction between time and treatment on mitotic activities was also highly significant ($F_{4,44}$=22.658, P<0.001). The figure shows that mitotic activities increased sharply from 4-6 in the control hamsters to 14.390 division/crypt ± 0.539 on day 28 and 15.050 division/crypt ± 0.354 on day 56 in hamsters given primary infection. Similar increases were also detected in the number of mitotic figures during trickle infection with a small dip of 9.23 division/crypt ± 0.31 on day 42 followed by a sharp and quick rise of 19.730 division/crypt ± 0.548 on day 56 and 20.22 division/crypt ± 1.75 on day 70. The increase in the observed mitotic activities with time across the data-set (r_s=0.850, n=15, p<0.001) was significant in the primary infected animals and (r_s=0.777, n=25, p<0.01) in trickle infected groups of hamsters but not in naïve animals (r_s=-0.208, n=15, p=0.457).

5.3.1.4 – Mast cell responses

As can be seen from Figure 5.5, mast cell numbers were elevated in both primary and trickle infected groups compared to control uninfected animals. Two-way ANOVA were performed and revealed that the main effect of time and treatment were significant ($F_{4,44}$=6.833, P<0.001 and $F_{2,44}$=17.011, P<0.001, respectively), with a significant interaction between treatment and time ($F_{4,44}$=5.600, P<0.01). The number of mast cells increased with time in trickle infected groups (r_s=0.859, n=25, p<0.001) but not in naïve control groups (r_s=-0.492, n=15, p=0.063). Although the number of mast cells was higher in primary infected groups compared with naïve uninfected hamsters, the changes of mast cells during primary infection with time was not significant (r_s=0.491, n=15, *p=0.063*).

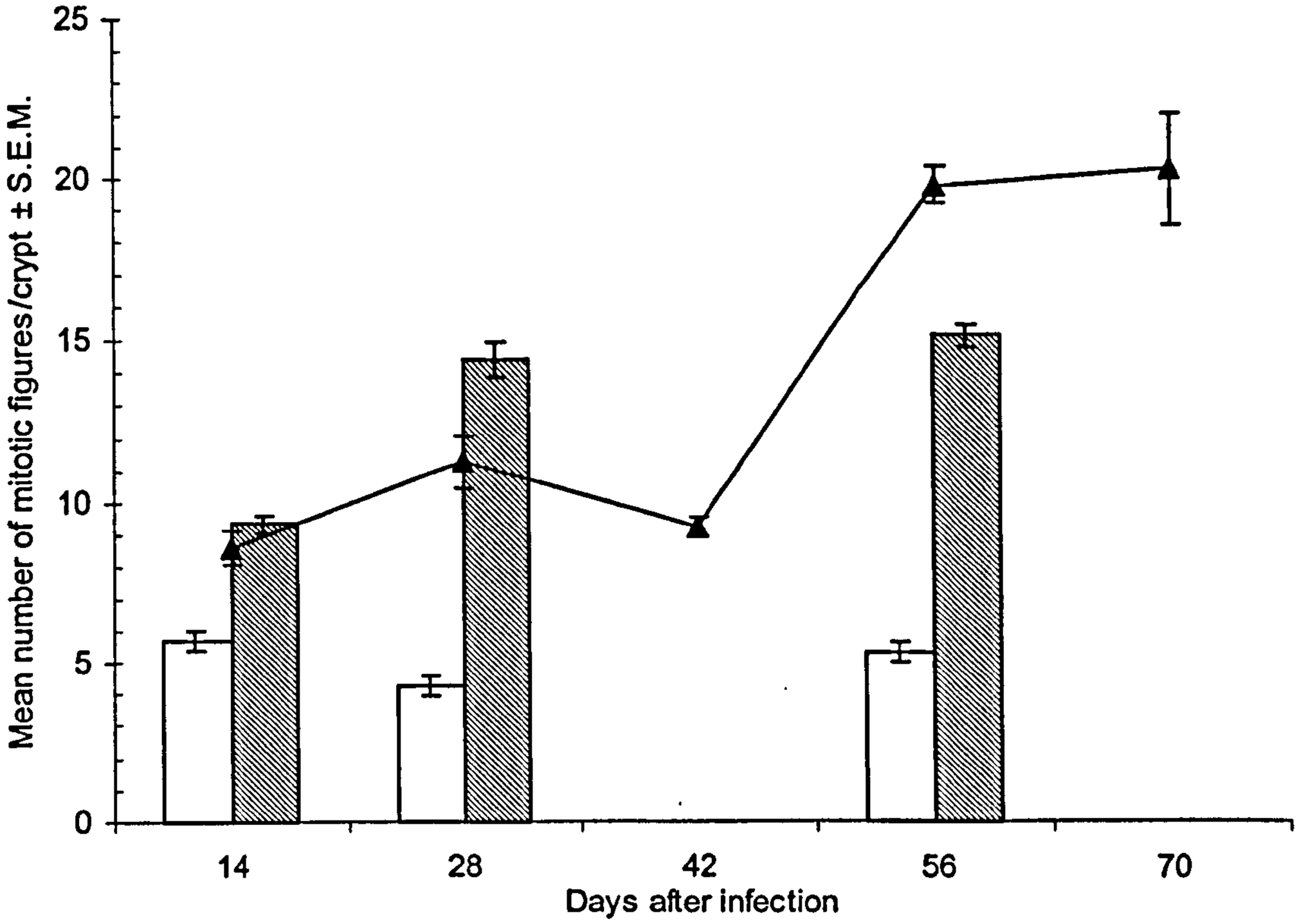


Figure 5.4 – Mean number of mitotic figures in the crypts of Lieberkuhn (±S.E.M.) in the intestinal mucosa of:

Naïve-uninfected hamsters (▭).

Primary infected hamsters with *A. ceylanicum* (▨).

Trickle infected hamsters with *A. ceylanicum* (–▲–).

Statistical analysis

Analysis of mitotic figure by 2-way ANOVA with treatment (3 levels, naïve, primary and trickle) and time (5 levels, 14, 28, 42, 56 and 70) revealed:

Main effect of time	$F_{4,44}$=58.408, P<0.001
Main effect of treatment	$F_{2,44}$=137.381, P<0.001
Interactions between treatment and time	$F_{4,44}$=22.658, P<0.001

Model R^2 = 0.923

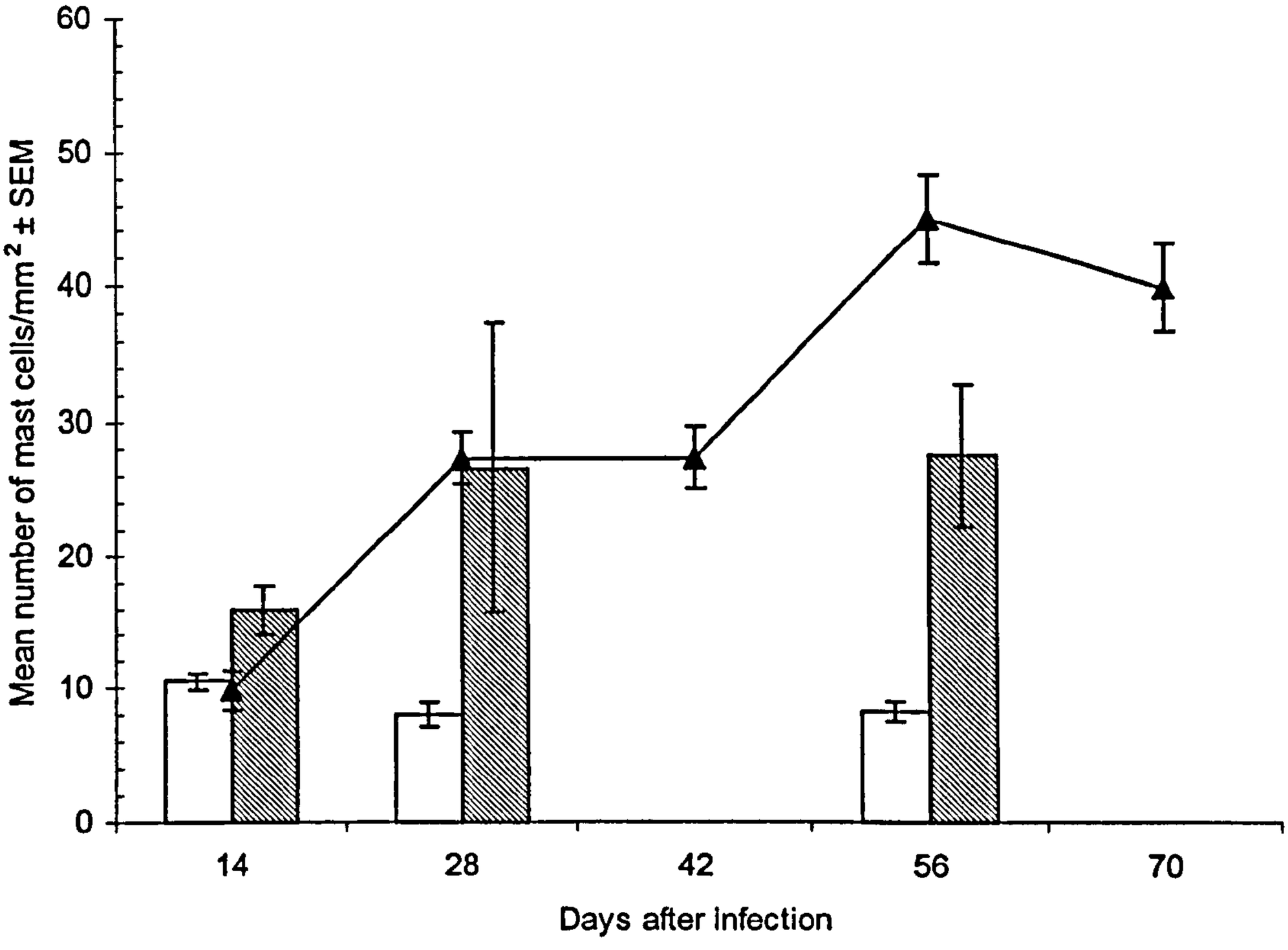


Figure 5.5 – Mean number of mast cells/mm² in the intestinal mucosa of:

Naïve-uninfected hamsters ( ).

Primary infected hamsters with *A. ceylanicum* ( ).

Trickle infected hamsters with *A. ceylanicum* ( ).

Statistical analysis

Analysis of mast cells by 2-way ANOVA with treatment (3 levels, naïve, primary and trickle) and time (5 levels, 14, 28, 42, 56 and 70) revealed:

Main effect of time	$F_{4,44}$=6.833, P<0.001
Main effect of treatment	$F_{2,44}$=17.011, P<0.001
Interactions between treatment and time	$F_{4,44}$=5.600, P<0.01

Model R^2 = 0.624

5.3.1.5 – Goblet cell responses

As can be seen from Figure 5.6, there was a sharp increase in the number of goblet cells from the mean of 48.340 cells/mm^2 ± 8.677 on day 14, which is similar to that found in the naïve group on the same day, to 253.120 cells/mm^2 ± 9.911 on day 28 and 281 cells/mm^2 ± 19.806 on day 56 in hamsters given the primary infection. This increase was significant with time (r_s=-0.813, n=15, p<0.001). At the same time, two peaks in the number of goblet cells were apparent on days 28 and 56 post-infection for groups that received trickle infection. This increase was significant with time (r_s=-0.813, n=15, p<0.001). Goblet cells increased from 104.4 cells/mm^2 ± 9.3 on day 14 up to 234.6 cells/mm^2 ± 18.0 on day 56 with a partial return on both days 42 and 70. Results from 2-way ANOVA analysis showed that time affected goblet cells significantly ($F_{4,44}$=23.414, P<0.001) and a similar significant main effect of treatment was also observed ($F_{2,44}$=62.818, P<0.001). The interaction between time and treatment was also highly significant ($F_{4,44}$=14.164, P<0.001).

5.3.1.6 – Paneth cell responses

Normal uninfected DSN hamster tissue stained with phloxine-tartrazine showed maximum numbers of Paneth cells. They were located only at the base of the crypts of Lieberkuhn. As can be seen in Figure 5.7, the Paneth cell numbers were much lower in both infections compared to those found in the tissues of the naïve groups. The decrease in Paneth cells with time during primary infection from 2.190 cells/crypt ± 0.381 on day 14 to less than 1 cells/crypt on day 56 was significant (r_s=-0.776, n=15, p<0.01). Although the number of Paneth cells in the primary infection declined significantly with time, there was no evidence that Paneth cells reacted similarly in either trickle infection or naïve hamsters. Despite the obvious differences between Paneth cell counts in naïve and trickle groups, both were not significantly affected with time (r_s=-0.026, n=25, p=0.904 and r_s=0.491, n=14, p=0.136, respectively). When 2-way ANOVA analysis was applied, Paneth cells were not affected

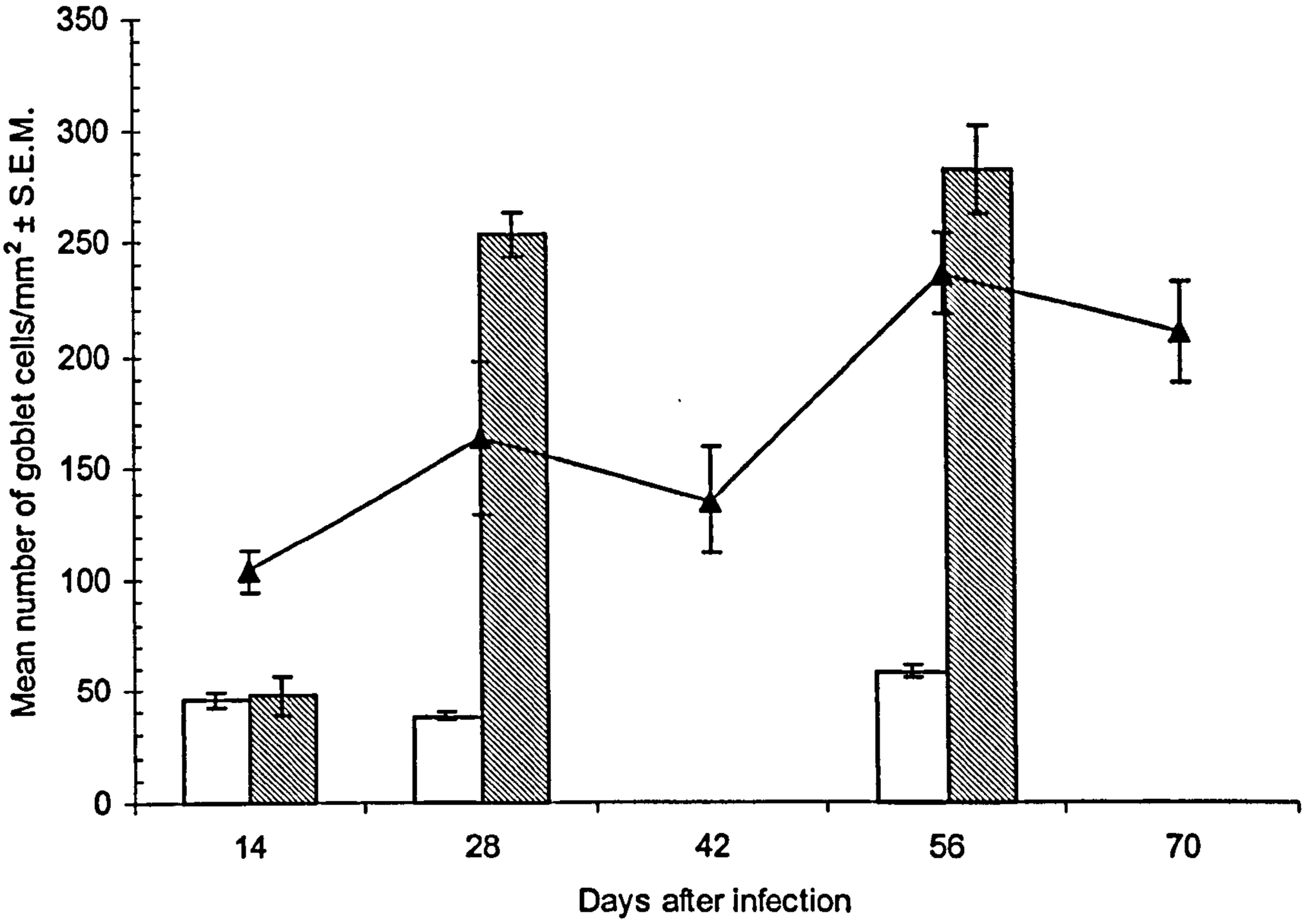


Figure 5.6 – Mean number of Goblet cells/mm² of the intestinal mucosa of:

Naïve-uninfected hamsters (▭).

Primary infected hamsters with *A. ceylanicum* (▨).

Trickle infected hamsters with *A. ceylanicum* (–▲–).

Statistical analysis

Analysis of goblet cells by 2-way ANOVA with treatment (3 levels, naïve, primary and trickle) and time (5 levels, 14, 28, 42, 56 and 70) revealed:

Main effect of time	$F_{4,44}$=23.414, P<0.001
Main effect of treatment	$F_{2,44}$=62.818, P<0.001
Interactions between treatment and time	$F_{4,44}$=14.164, P<0.001

Model R^2 = 0.835

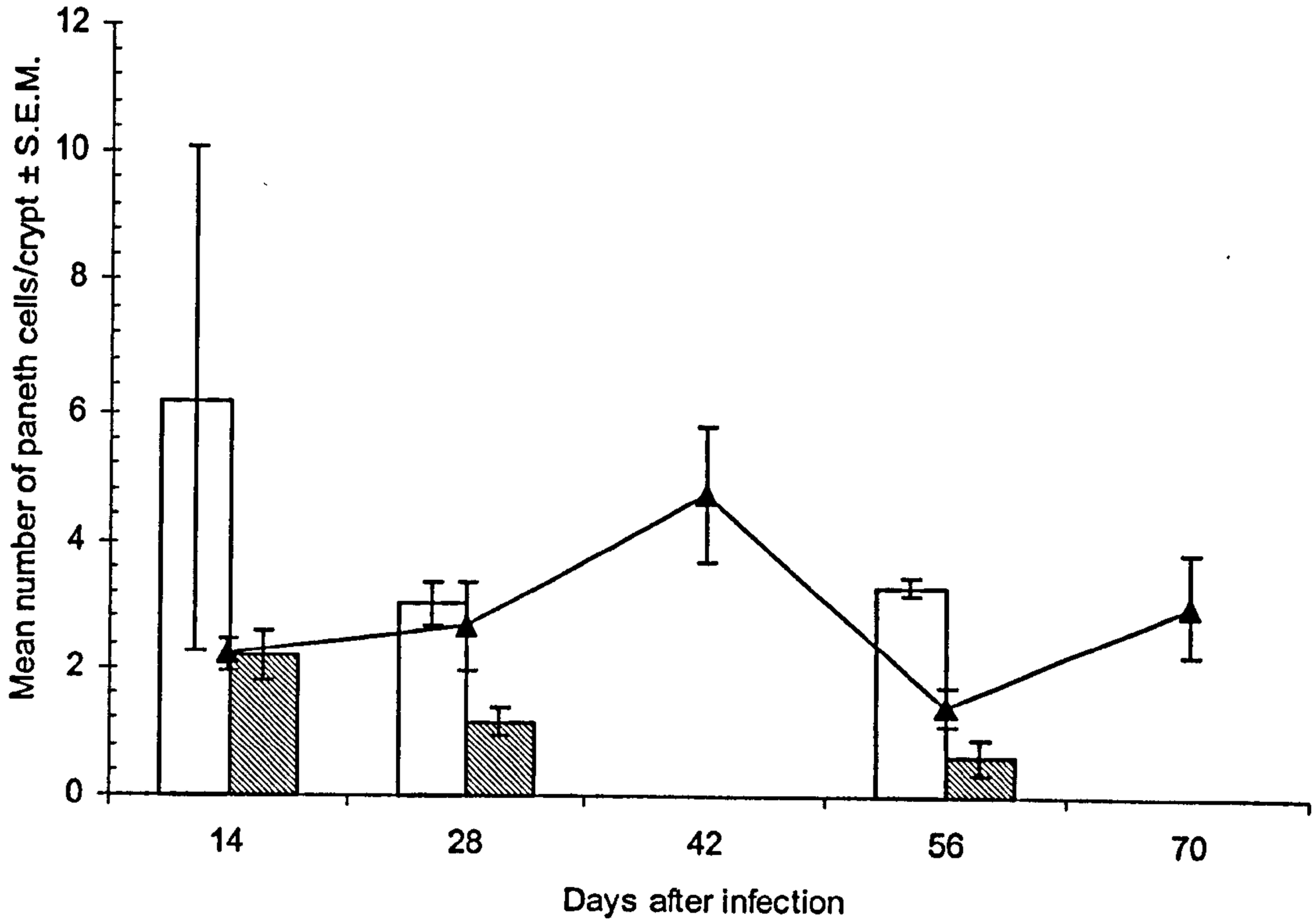


Figure 5.7 – Mean number of Paneth cells/crypt (± S.E.M.) in the intestinal mucosa of:

Naïve-uninfected hamsters (▭).

Primary infected hamsters with *A. ceylanicum* (▨).

Trickle infected hamsters with *A. ceylanicum* (–▲–).

Statistical analysis

Analysis of Paneth cells by 2-way ANOVA with treatment (3 levels, naïve, primary and trickle) and time (5 levels, 14, 28, 42, 56 and 70) revealed:

Main effect of time	$F_{4,44}$=1.431, P=0.240
Main effect of treatment	$F_{2,44}$=3.930, P<0.05
Interactions between treatment and time	$F_{4,44}$=0.494, P=0.740

Model R^2 = 0.092

significantly with time ($F_{4,43}$=1.431, P=0.240) and the interaction between treatment and time was also not significant ($F_{4,43}$=0.494, P=0.740). However, a significant main effect of treatment was found ($F_{2,43}$=3.930, P<0.05).

5.3.1.7 – Eosinophil responses

Figure 5.8 summarises changes in eosinophil numbers in the mucosa of hamsters infected with *A. ceylanicum* and the statistical analysis is given in the legend. There was a highly significant effect of treatment and as can be seen both infected groups had elevated numbers compared to the naïve control hamsters on all the days examined. In the naïve groups of hamsters, the number of eosinophils were relatively low and ranged between 58-190 cells/ mm^2 whereas they increased sharply to a peak of 763.1 cells/mm^2 of tissue ± 252.7 on day 28 followed by a decline to 341.9 cells/mm^2 of tissue ± 46.1 on day 56 in primary infected groups of hamsters. In contrast, eosinophils increased gradually from 390.194 cells/mm^2 of tissue ± 163.8 on day 14 to 785.1 cells/mm^2 of tissue ± 186.1 on day 56 but dropped back to 628.080 cells/mm^2 of tissue ± 91.2 at the end of experiment. Neither ANOVA nor analysis by Spearman test revealed significant time dependant changes in this experiment ($F_{4,42}$=1.501, P=0.219), (naïve r_s=-0.305, n=14, p=0.289, Primary r_s=-0.472, n=15, p=0.075, and trickle r_s=-0.263, n=24, p=0.214). Also there was no significant interaction between time and treatment in this experiment ($F_{4,52}$=1.298, P=0.286) but there was a significant main effect of treatment ($F_{2,42}$=6.256, P<0.01).

5.4 – DISCUSSION

The experiment reported in this chapter confirms and extends the finding of the previous chapter on host-parasite relationship of *A. ceylanicum* in hamsters. It shows very clearly that changes which occur in the intestine of hamsters infected with *Ancylostoma ceylanicum* during a single pulse primary

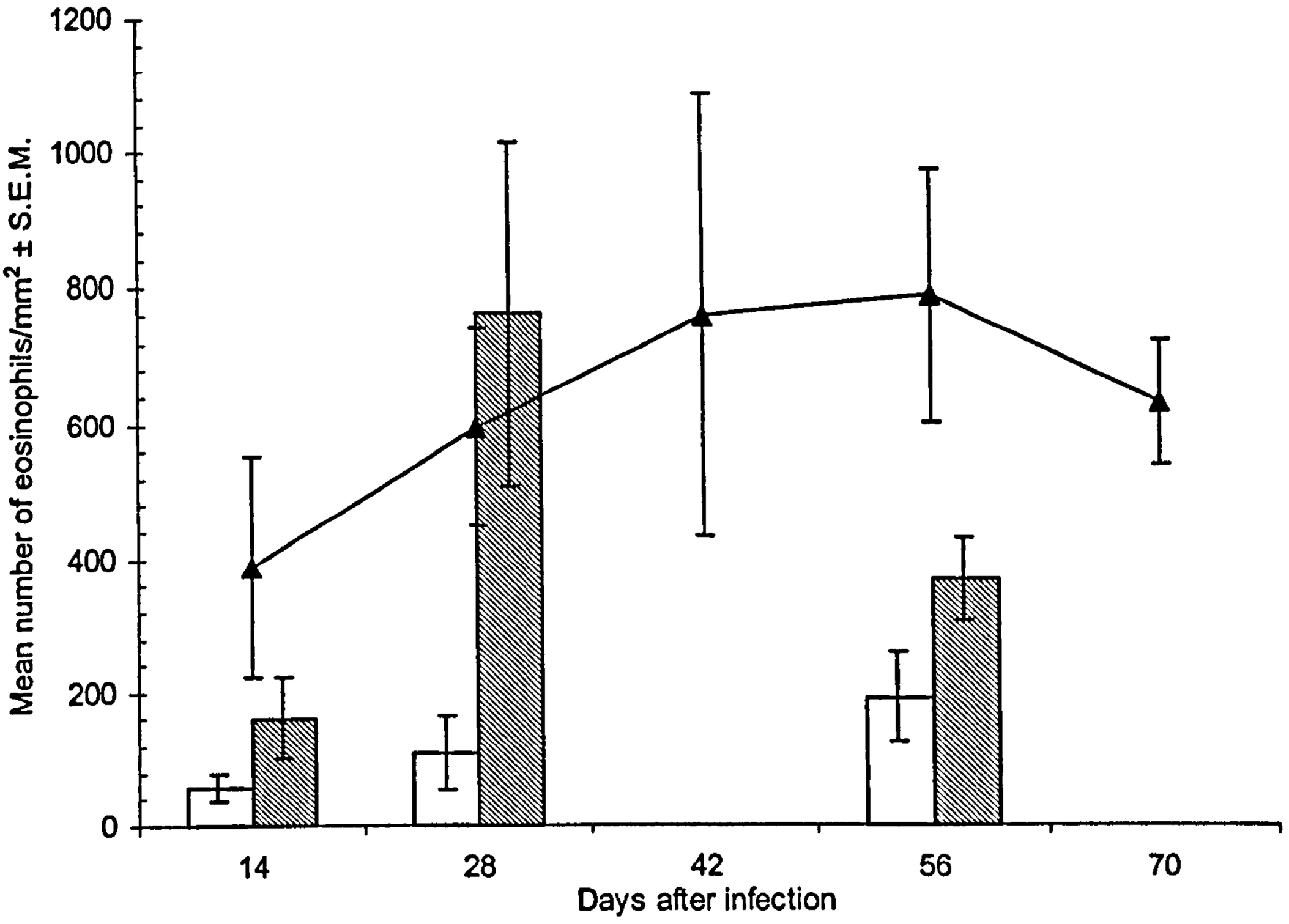


Figure 5.8 – Mean number of eosinophils/mm² of the intestinal mucosa of Naïve-uninfected hamsters (▭).

Primary infected hamsters with *A. ceylanicum* (▨).

Trickle infected hamsters with *A. ceylanicum* (▲).

Statistical analysis

Analysis of Eosinophils by 2-way ANOVA with treatment (3 levels, naïve, primary and trickle) and time (5 levels, 14, 28, 42, 56 and 70) revealed:

Main effect of time	$F_{4,42}$=1.501, P=0.219
Main effect of treatment	$F_{2,42}$=6.256, P<0.01
Interactions between treatment and time	$F_{4,42}$=1.298, P=0.286

Model R^2 = 0.276

infection can also occur with some remarkable patterns during continuous exposure and that some of these differ markedly. These changes which included cellular proliferation, infiltration of the mucosa by inflammatory cells, up regulation of goblet cells and marked alteration to intestinal architecture were reported here for the first time. To my knowledge, there has not been any previous published work on these types of responses in animals exposed to trickle infection with hookworms. The experiment was designed to bridge the gap between the somewhat artificial single pulse primary infection often used for convenience in laboratory-based animal work and naturally acquired infection in the field where man and animals are continuously exposed to frequent challenges.

Inflammatory changes observed during the course of infection with GI nematodes that cause self-limiting infection, terminated by expulsion of the parasites, have been documented thoroughly in the literature. However, far less is known about such changes in animals harbouring chronic infections in which worms are not expelled within 2-3 weeks of infection. Even those which have been reported in *H. polygyrus* (Behnke *et al.*, 1992; Behnke *et al.*, 1987) and hookworms (Behnke *et al.*, 1994; Sarinas and Chitkara, 1997) have generally utilised single pulse primary infections, which it can be argued, are artificial and not frequently encountered in nature. The experiment reported in this chapter was designed to readress the balance by documenting infection in frequently infected animals by administering a trickle infection.

The experimental design included a group of hamsters exposed to a single pulse primary infection for comparison. Establishment of worms was approximately less than 25% of the total number of 50 larvae administered and fell with time which was much in line with some earlier data such as illustrated in chapter 3 figure 3.1.1 (A), and although there was no statistically significant reduction in worm burden, there was some indication of worm loss throughout the experiment (r_s=-0.493, n=15, P=0.062). As reported earlier (Brailsford and Behnke, 1992a), worm burdens in trickle infected hamsters stabilised from the outset and there was no accumulation of worms with successive weeks after initial exposure. This suggests that right from the outset, subsequent dose of larvae were being resisted whilst the initially established worms survived. In

time there was evidence of a net loss of worm that was significant. Therefore, one group of hamsters were unable to clear worms and at best showed only a slight reduction whilst the other showed significant loss of worms during the period of the experiment and resisted incoming additional larvae.

5.4.1 – Architecture changes in the intestinal mucosa

As reported in the previous chapter, changes in intestinal architecture, characterised by atrophy in the villi, crypt hyperplasia and cellular proliferation in the crypts of Lieberkuhn were associated with *A. ceylanicum* infection. These changes showed similarity of those found in some other species, such as *T. spiralis* (Kamal, 2001; Menson-Smith *et al.*, 1979) and *Giardia* (Levinson and Nastro, 1978). It was also found that these changes were associated with a continuous dosing of *A. ceylanicum* larvae in hamsters, supporting previous work of an association between changes in the architecture of the intestinal mucosa and infection during trickle infections with nematodes (Coop *et al.*, 1979; Jackson *et al.*, 1983).

Experiments in sheep continuously infected with *T. vitrinus* have shown very clearly that a number of changes occur including atrophy and flattening in villus structure together with infiltration with lymphocytes. Some lesions in the tissues were associated with loss of worm burdens during trickle infection. However, control groups of sheep, not exposed to infection, did not show such changes. Similar work has been conducted using *T. colubriformis* and this concluded that morphological changes in the intestine of lambs occur as a consequence of infection (Coop *et al.*, 1979). Examination by scanning electron microscopy showed areas devoid of villi, with numerous worms embedded in the affected areas. If changes in the mucosa are always associated with the presence of the worm as a consequences of the worm feeding activities then we might expect that changes in the intestinal mucosa and cellular infiltration increase as worm burdens increase too. However, besides parasite numbers, other factors such as the method of attachment and feeding, and the antigenic

stimulus, which alter between species, may complicate matters and do not allow straight forward comparison between species without extreme caution.

In the experiment presented in this chapter, the usual range in villous height in naïve, uninfected hamsters was from 1044 to 1091 µm and this is in agreement with values from the experiment reported earlier in Chapter 3 (Experiment 4 ranging from 1060-1093.5 µm) (Figure 3.10). It also confirmed that this height begun to decline by approximately 50 % of its length within two weeks following initial infection and the continuous decline with time after infection was comparable to that found in data from previous experiments, such as those reported in chapter 3, figure 3.10 (B), which showed that villous height declined with time after infection.

It is very clear from the results in figure (5.3) that the height of the villi declined continuously in hamsters exposed to trickle infection over the period of this experiment and it showed a similar reduction to that observed in the primary infected groups. However, decline in villous height was marginally less among trickle infected hamsters throughout the experiment compared to the primary infected groups. It was expected that hamsters subjected to the trickle dose of infective larvae of *A. ceylanicum* would show more atrophy in villous height compared to primary infection as a consequence of worms accumulating in the intestine. However, the results from this chapter suggest that worms from the initial dose established and that there was no net accumulation of worms throughout the experiment. It was also found that some stages of *A. ceylanicum* such as L4 were recovered from the intestine, but no L3 stage were observed. The number of worms recovered from each stage was similar to the worm burden reported by Brailsford *et al.* (1992a) who found that all stages of *A. ceylanicum* could be detected in the hamster's intestine repeatedly infected with *A. ceylanicum* after two weeks following initial infection. However, L3 stages were not recovered in this experiment. This suggests that anti-establishment immunity may have developed early in the experiment, although the possibility that L3 were missed has to be taken into account.

When a direct comparison was made between trickle and primary infected hamsters, it was evident that the villi in the trickle infected groups during all 3 times after infection when direct comparison was possible were slightly higher.

This may simply reflect the differences in worm burden between trickle and primary infected groups. Some support for this can be found in the dose response experiment reported in Chapter 3. Villous atrophy in hookworm infections may be partially due to the immune response, but worm feeding activities may equally contribute to this. Since, hookworms browse on the mucosal epithelium, heavy infections are more likely to cause severe damage to villi than lighter ones.

The experiment also revealed a concurrent increase in the depth of the crypts and in mitotic activity in the enterocytes of the mucosa. These increases have characteristic patterns which are most likely to be directed by Th2 mediated mucosal responses rather than feeding activities. The results presented in Figure (5.3) show a steady increase in the crypt depth of both treatments with duration of infection. However, values from day 28 and 42 of the trickle infected groups were similar.

In the experiment reported here, the survival of worms in the intestine was considerably longer than, for example, in *T. spiralis* or *N. brasiliensis* (Behnke *et al.*, 1994; Ferguson *et al.*, 1980) and consistent with earlier reports of chronicity of single pulse primary infection with hookworm in hamsters (Garside and Behnke, 1989). However, there was evidence of a slow loss of worms, especially in trickle infected animals, again in line with earlier research (Brailsford and Behnke, 1992a). The remarkable feature of this experiment was that so many worms were able to survive despite the marked changes to the mucosal architecture, including the almost complete loss of villi as the infection progressed. Loss of worms was more marked in trickle infected hamsters despite less marked reduction in villous height although crypt depth was actually greater than in hamsters with the primary infection. Trickle infected hamsters also had fewer worms. These results suggest that whilst villous height is probably reduced by both the feeding activities of the worm and the mucosal response to infection, crypt depth may simply be a reflection of the intensity of the host response. Thus, the more marked changes in crypt depth in trickle infected hamsters may reflect a more intense protective response with greater loss of worms from trickle compared with primary infected hamsters.

5.4.2 – Mast cells

The intestinal mucosa of naïve, uninfected animals generally has very few MMC evident, seldom exceeding 12 cells/mm^2 of intestinal tissue (See Chapter 3, figure 3.14 (B) and figure 3.24 (C) and in the present experiment the mean value recorded ranged from 8 to 10, which is within the usual range.

When animals were exposed to a primary infection, a rise was already evident on day 14 and the MMC density rose above 25 cells/mm^2, more than twice above that in naïve hamsters. This compares well to earlier experiments when the number of MMC was around 22 (Chapter 3, Experiment 4). As in previous chapters, the MMC density plateaued and was markedly elevated until day 56 post infection. The correlation of MMC with time was not significant, although close to significance (r_s=0.491, n=15, P=0.063) because of the variances between animals and because MMC numbers were already elevated on day 14. Had the values obtained for naïve hamsters on day 14 been included as representing a baseline on day 0, the correlation coefficient would have been (r_s=-0.721, n= 20, P<0.001).

In contrast to hamsters given a single pulse primary infection, MMC numbers rose higher in trickle infected animals and achieved a higher plateau towards the end of the experiment. This was despite fewer adult worms and a consistent and significant fall in worm numbers from day 14 onwards. The different pattern of mastocytosis in the hamsters subjected to single pulse primary and trickle infection suggests that it is not only the adult worm burden that provides the stimulus for mastocytosis. Two alternative explanations are suggested. Firstly, in trickle infected animals, it may be the additional stimulus provided by incoming L3 that helps to drive mastocytosis. Additionally, the loss of adult worms from trickle infected hamsters suggests that under this infection protocol, better protective immunity has developed, perhaps boosted by continuous re-exposure to L3. It is possible that the intestinal mastocytosis in these animals reflected a combination of events including a mastocytosis sufficiently intense to be responsible for worm loss. However, this explanation is not compatible with the evidence that adult worm burden were declining from

day 14 onwards when MMC density was similar to that in animals subjected to the single pulse primary infection.

Histological evidence that MMC numbers are elevated, however, does not provide certainty that they are fully functional and capable of realising their effectors. An avenue for future work will be to measure the activity of mucosal mast cells proteases, as has been done in mice and rats. Changes in MMC response and intestinal mucosal levels of MMC would provide support for their activities.

4.4.3 – Goblet cells

As in previous experiments (Chapter 3 and 4), goblet cell numbers in naïve, uninfected hamsters did not change during the course of this experiment and the mean values remained below the 60 cells/mm^2 , much as in earlier work (Chapter 3 of Experiment 7, figure 3.3.6).

Equally, the primary response was dramatic with an approximately 4 fold increase by day 28 and this was maintained until day 56. This was in agreement with results reported earlier (Experiment 4 in Chapter 3) when goblet cell numbers in the intestinal tissue were very similar on day 28. However, on day 14 of this experiment, the number of goblet cells were found to be similar to those counted in naïve hamsters, which contrasts with the findings in Chapter 3 (Fig 3.1.5-B) where the number of goblet cells in primary infected hamsters were double those observed in naïve hamsters on the same day. In Experiment 4 (Chapter 3) goblet cell numbers continued to increase towards the end of the experiment even when there was not much difference in the total number of worms established in the intestine (approximately 19 worms in experiment 4 in Chapter 3 and 16 worms in this experiment).

In contrast, the goblet cell hyperplasia began earlier in trickle infected hamsters and never exceeded that seen in animals given the single pulse primary infection. Thus, a contrasting pattern emerged suggesting that repeated infection may accelerate the onset of goblet cell hyperplasia. Since the data in

Chapter 3 showed that the goblet cell response was dependant on adult worm numbers, the lower plateau obtained in trickle compared with single pulse primary infected hamsters may simply reflect fewer adult worms in the former group. Moreover, fewer adult worms on day 42 might explain the reason for goblet cell numbers being lower on that day.

5.4.5 – Paneth cells

The naïve control groups of hamsters killed on day 14 had somewhat higher numbers of Paneth cells than expected. The group killed on day 28 and 56 were more typical in this respect with means of 3 and 3.6 respectively. Throughout this type of experiments, the usual range in naïve hamsters was 3-6 cells/crypt.

In line with earlier experiment (Chapter 3, figures 3.2.7 and Chapter 4, figure 4.1.6), Paneth cells fell in animals harbouring the primary infection. Moreover, in complete contrast, they were maintained at approximately normal levels in trickle infected hamsters. The only exception was on day 56 when the levels dipped.

Thus, there was an interesting contrast between single pulse primary and trickle infection with regard to Paneth cell responses. It is difficult to see an obvious explanation, but if the fall in Paneth cell numbers normally seen following *A. ceylanicum* infection in hamsters is driven by the activities of adult worms, then it may be that continuous exposure to larvae helped to up regulate Paneth cell numbers. In other word, lack of significant changes in Paneth cell numbers with time after infection may be explored by the outcome of two processes which counter balance one another: adult worms lower Paneth cell number whilst continuous exposure to larvae raises the number. One way to test this hypothesis would be to subject hamsters to trickle infection and intermittently treat them with an anthelmintic capable of removing adult worms only. Pyrantel may be suitable for this purpose as it causes spastic paralysis

followed by passive elimination of the adult worms (Aubry *et al.*, 1970). This would help to clarify whether Paneth cell numbers are affected by larvae.

5.4.6 – Eosinophils

The results reported in this chapter revealed a dramatic increase in the number of eosinophils in hamsters receiving primary infection compared to naïve-uninfected groups. This was a confirmation of previous findings in Chapters 3 and 4. The number peaked around 800 cells/mm^2 by day 28 followed by a drop in eosinophil numbers to approximately half by day 56 pi. Data from murine ancylostomiasis by Gowri, *et al.* (1992) showed that eosinophils in blood begun to increase in primary infection from the first day to reach the peak on day 4 pi while the peak was delayed until three weeks post infection and sustained with thereafter, a slight decrease towards day 30 after exposure to repeated infections. It was also found by Kolhe, *et al.* (1983) that Swiss albino mice immunized with *A. caninum* had higher numbers of peripheral eosinophils compared to naïve uninfected mice with peaks seen on day 16 of the primary infections and 4 and 9 days after infection in immunized mice.

As can be seen from Figure 5.8 the number of eosinophils in trickle infected hamsters increased with time to levels higher than those observed in primary infected groups. It was suggested by Balic, *et al.* (2000) that adult worms are weaker at inducing eosinophilia compared to early stages. Therefore, the continuous exposure to the strong incoming stimulation by larvae could be the reason that tissue eosinophil numbers were much higher in trickle infected hamsters where animals were exposed to larval stage more frequently than those exposed to a single dose of infection.

The experiment reported in this chapter is unique in as far as it is the first trickle infection experiment with hookworms in which the mucosal response has been documented in such details. Although continuous exposure to hookworm larvae elicited marked changes in several respects, these differed from the typical changes associated with single pulse primary infection. Some of these

differences could be accounted for by the lower adult worm burdens following trickle infection but not all. Where the response was more intense (e.g. mast cells), it is likely that it was driven in part by the regular arrival of a few larvae providing reinforcement of the signals that elicited the response.

Overall the experiment reported in this chapter support the hypothesis proposed that changes in the intestinal mucosal immunity during trickle infection with hookworms differ from those observed during primary infection. These differences may provide opportunities for further experimentation to explore the nature of the signals that elicit immunity to hookworm. In time, knowledge of these may provide a better understanding of the reason for chronic survival of hookworms in most subjects exposed naturally to infection.

CHAPTER SIX

THE EFFECT OF TREATMENT WITH CYCLOSPORIN A (C_SA) ON THE MUCOSAL RESPONSES OF HAMSTERS INFECTED WITH *(A. CEYLANICUM)*

6.1 – SUMMARY

An experiment was carried out to assess the effect of treatment with Cyclosporin A (CsA) on the mucosal response to the hookworm *Ancylostoma ceylanicum*. Unexpectedly, treatment with CsA affected worm establishment and survival and this had consequences for the interpretation of data. Nevertheless, CsA treated, infected animals showed weaker goblet cell responses and less marked changes in intestinal architecture than untreated infected animals. However, there was no difference in mast cell numbers. Although Paneth cell numbers dropped in both treated and non-treated infected animals by day 14, they rebounded markedly by day 22 in treated infected animals. Mucosal eosinophil counts were almost completely suppressed in treated infected animals as on day 14, but rebounded with a prominent response by day 22. These results suggest that CsA had some effect in hamsters and modified the mucosal response, but interpretation was confounded by the concurrent anthelmintic effect of this drug.

6.2 – INTRODUCTION

The small intestine is very important to the host as the principal site for the uptake of the breakdown elements produced by digestion. However, intestinal tissue has a relatively fast rate of turnover and lesions are rapidly repaired (Castro *et al.*, 1990). Such lesions and associated changes are commonly encountered during parasitic infections including those caused by hookworms (Carroll *et al.*, 1986; Chaudhuri and Saha, 1964; Ferguson *et al.*, 1980; Garside *et al.*, 1990; Mukerjee *et al.*, 1988). Alteration in the intestinal architecture, such as villous atrophy and crypt hyperplasia, are known to occur during hookworm and some other parasitic infections (Carroll *et al.*, 1986; Duncombe *et al.*, 1978; Ferguson *et al.*, 1980). Moreover, intense changes and cellular infiltration of the mucosa is also characteristic of hookworm infection (Garside *et al.*, 1990; Gorski *et al.*, 1999; Migasena *et al.*, 1972). Canine hookworms, such as *A.*

caninum, were found to cause abnormal villous patterns manifested by villous atrophy and crypt hyperplasia during moderate and heavy administration of larvae with associated increases in goblet cell activities (Migasena *et al.*, 1972). Similarly, dogs infected with *A. ceylanicum* showed severe villous atrophy with associated infiltration by neutrophils and eosinophils around the site of worm attachment (Carroll *et al.*, 1984; Carroll *et al.*, 1985). Moreover, the epithelium of the human intestine was flattened, villi were absent and a dense cellular infiltration in the *lamina propria* was observed during infection with *A. duodenale* (Burman *et al.*, 1970). Mild cellular infiltration into the intestine of mice infected with *A. caninum* was noted by Banerjee, *et al.* (1970) and further qualified by Vardhani and Johari (1978) who reported increases in mast cells in first week of infection at a time when larvae would have been expected to stimulate the response of mast cells. It was believed by Chaudhuri and Saha (1964) that the changes that occur in the intestine during hookworm's infection lead to intestinal disorder, abdominal pain and discomfort to the host. The physical damage in the intestinal structure, concentrated at the feeding site during hookworm infection, may also arise from the direct effects of the worms' feeding activities where adult worms bite into the gut tissue (Verma *et al.*, 1968).

It has been shown that altered cellular activities in the intestine during GI nematodes infections are mainly controlled by cytokines. These controlling influences are exerted from the activated T cells. Now it is known that the type two (Th2) responses are vitally important in driving the intestinal inflammatory responses, which result in worm expulsion (Else, *et al.*, 1998). The requirement of T cells in the immune mechanisms controlling worm expulsion has been demonstrated using hypothymic nude mice (Jacobson and Reed, 1974). It was reported that the expulsion of some *Strongyloides spp.*, such as *S. ratti* and *S. venezuelensis*, from hypothymic nude mice were entirely T cell dependant (Abe and Nawa, 1988; Sato and Toma, 1990). However, work by *Abe, et al.* (1992) showed that treatment of nude mice (nu/nu) with the cytokine IL-3, one of the cytokines secreted by Th2 cells, restores the capacity to reject *S. ratti* but not *N. brasiliensis* because it supports mastocytosis. Other studies showed that the rejection of nematodes, as seen in mice infected with *N. brasiliensis*, from the intestine were associated with cellular responses, such as mastocytosis and

goblet cells hyperplasia (Nawa *et al.*, 1994) and the effectors had different effects on the different species (e.g. the mast cell response is important in resistance to *T. spiralis* whereas the mucus response is an eventual component required for the expulsion of *N. brasiliensis*).

The importance of T cells which initiating the host protective immune responses against nematode infections have also been supported by many different experimental approaches. These include interference with T cells signalling by the use of several types of immunosuppressive drugs, using animals with key genes controlling elements of the immune response knocked out by gene manipulation or antisera to key component. However, since the last two mechanisms are not available for experiment in hamsters, it was decided to use a drug known to affect T cell function in mammals.

Several different types of immunosuppressive agent can be used to affect T cells, one of which is Cyclosporin A, a natural hydrophobic cyclic polypeptide consisting of 11 amino acids. The mechanism of its action has been investigated and studied extensively (Schreiber *et al.*, 1993; Schreiber and Crabtree, 1992). CsA inhibits T cell activation by inhibiting the intracellular route of signalling following ligation of the T cell receptor. Recent studies of the immunosuppression indicated that the immunosuppressive action of CsA in some helminth and protozoan infections depends on the drug binding to the intercellular receptor cyclophilin (CyP). The use of CsA treatment in mice infected with R. microstoma or R diminuta revealed the contribution of T cell-dependent mechanisms (Mclauchlan *et al.*, 1999). CsA prevented worm expulsion, permitting some individual worm to reach maturity, and abrogated mast cell proliferation and mast cell protease 1 production and release. Similarly, studies on protozoan parasites, such as *Leishmania major*, have indicated that the immunosuppressive effects are mediated primarily by binding to (CyP) (McLauchlan *et al.*, 2000; Pahl *et al.*, 2002).

In addition to its successful immunosuppressive action, this drug also has been shown to have some direct effects on the parasites invading the host (Bolas_Fernandez *et al.*, 1988; Chappell *et al.*, 1989; Hurd *et al.*, 1993; Nilsson *et al.*, 1985). It was found that the anthelmintic effects of CsA target early

stages during development of parasite, affecting juvenile worms, as in the case of *Rodentolepis microstoma* and *Hymenolepis diminuta* (Chappell *et al.*, 1989). It has also been found that adult worms respond to treatment with CsA by a significant loss of weight. However, the anthelmintic action is not always apparent and data by Chappell, *et al.* (1989) showed that *H. diminuta* in the rat was completely unaffected by treatment with CsA. Data by Hurd, *et al.* (1993) have also indicated that the efficacy of the CsA against tapeworm infections in laboratory models is variable. They reported that when mice were given consecutive daily doses of CsA, beginning 2 days prior to infection with *Echinococcus granulosus,* significant reduction in the establishment of the hydatid cysts (measured in terms of cyst masses, cyst numbers and cyst wet weights) were observed. However, when the drug was administered 18 weeks post-infection, no significant reductions were recorded in the parameters measured. Therefore, the anthelmintic properties of CsA may vary between parasite species as well as different hosts.

The experiment described in this chapter was carried out to evaluate the effect of the immunosuppressive agent CsA on the mucosal response of hamsters to infection with *A. ceylanicum.* As in earlier chapters, the mucosal response to infection was quantified by the changes in the architecture of the mucosal surface (villous height and crypt depth) and by changes in the numbers of inflammatory cells in the mucosa. Since our hypothesis is that these changes are directed by T cells triggered by the antigens presented by hookworms, it was hoped to block the pathway of T cell activation using CsA treatment in order to establish the extent to which the changes in the intestine are controlled by T cell activity.

6.3 – EXPERIMENTAL DESIGN AND RESULTS

Forty adult female DSN hamsters were used in this experiment and were divided into four different treatment groups as shown in Table 6.1 according to treatment (Not infected nor treated, treated with CsA but not infected, treated with CsA and infected, infected but not treated with CsA). Five hamsters from

Table 6.1 – Experimental design and worm recovery in an experiment to assess the effect of Cyclosporin A on the intestinal mucosal immunity of hamsters infected with *A. ceylanicum*.

No. of hamster	Dose of L3 given	Dose of CsA given	Mean no. of worms recovered	Day killed
5	Nil	Nil	Nil	14
5	Nil	100mg/kg*	Nil	14
5	60 L3	Nil	20.2 ± 3.92	14
5	60 L3	100mg/kg*	2.4 ± 0.87	14
5	Nil	Nil	Nil	22
5	Nil	100mg/kg*	Nil	22
5	60 L3	Nil	10.4 ± 1.86	22
5	60 L3	100mg/kg*	3.2 ± 1.07	22

* Each hamster injected subcutaneously with 100mg/kg of CsA two days before the day of infection and then continued daily until day 9 P.I. and subsequently, second day until autopsy.

each treatment group were killed 14 days after infection and five on day 22. In practice, animals were maintained in groups of 2 and 3 to comply with new home office regulations. All hamsters were given Emtryl and Terramycin in drinking water one week prior to infection. Hamsters from all groups were injected subcutaneously with SRBC (Sheep red blood cells) and the blood was taken from their hearts at autopsy for Haemagglutination analysis.

6.3.1 – Total worm recovery

Differences in worm burdens between different treatments and on different days (14 and 22) after infection were analysed statistically and are illustrated in Figure 6.1. Analysis by 2-way ANOVA following transformation of worm burden data shows that there was a highly significant main effect of treatment on worm numbers ($F_{1,16}$=39.929, P<0.005). However, worm burden did not vary with time (Main effect of time $F_{1,16}$=0.605, P=0.488) and there was no significant interaction between time and treatment ($F_{1,16}$=2.247, P=0.153). As can be seen in (Figure 6.1), worm burdens declined between days 14 and 22 after infection in non-treated infected hamsters. However, the worm burdens in the group of hamsters that received a primary infection and were treated with CsA, showed lower worm burdens compared to those in infected not treated hamsters and there was no indication of any loss between days 14 and 22 (Figure 6.1).

The Haemagglutination test, which was used to detect the antibodies to the blood cells, failed to detect any response in treated and non-treated (CsA) animals injected with SRBC. Thus at the dose employed SRBC did not evoke an antibody response as expected.

6.3.2 – Assessment of intestinal pathology

Enteropathy of the small intestine in hamsters was observed and quantified by villous/crypt measurements to determine the effect of the

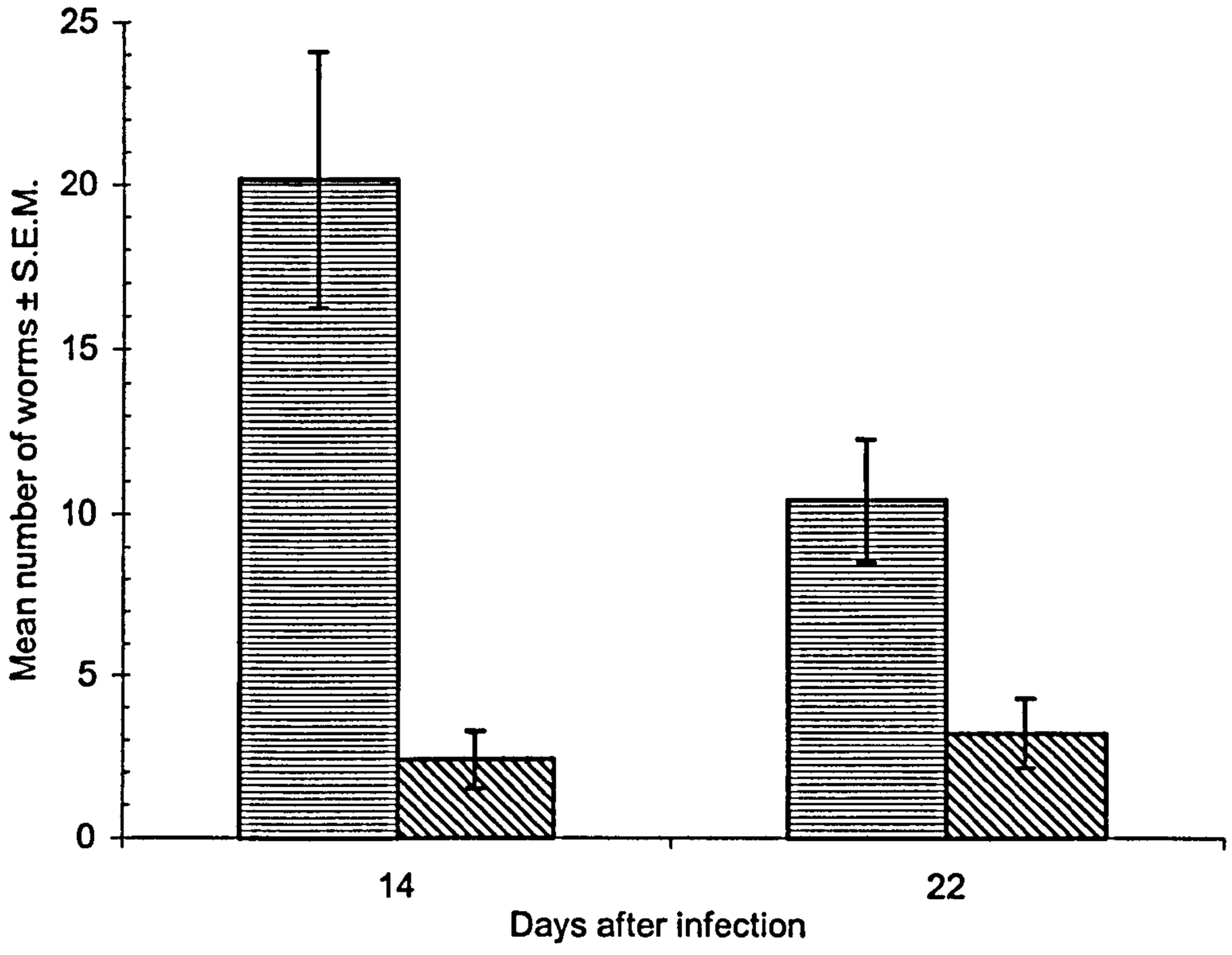


Figure 6.1 – Mean number of *A. ceylanicum* worm recovered (±S.E.M.) from the small intestine of:

Primary infected hamsters ( ).

Primary infected hamsters + CsA treatment ( ).

Statistical Analysis:

Analysis of these transformed data (Log X+1) by 2-way ANOVA with treatment (2 level, primary infected and primary with CsA treatment) and time (2 levels, 14 and 22) revealed:

Main effect of treatment	($F_{1,16}$=32.737, P<0.001)
Main effect of time	($F_{1,16}$=0.605, P=0.448)
Interaction between treatment and time	($F_{1,16}$=2.247, P=0.153)

Model R^2 = 0.632

Cyclosporin A on intestinal architecture of the hamsters. As can be seen in Figure 6.2, villous height remained relatively high with a mean height of 970µm ± 18.4 on day 14 and 957µm ± 29.2 on day 22 in the group receiving no infection and no treatment. The mean crypt measurements on both days from the same treatment were 123 µm ± 5.4 on day 14 and 138.8 µm ± 5.6 on day 22. On the other hand, villous atrophy and increased crypt depths were noted in both infected hamsters and those infected and treated with CsA. Two-way ANOVA revealed that there was a significant main effect of time and treatment on villous height ($F_{1,32}$=13.580, P<0.05 and $F_{3,32}$=52.750, P<0.001, respectively). Moreover, a significant interaction between time and treatment was also found ($F_{3,32}$=7.536, P<0.05). Analysis also revealed highly significant main effects of time and treatment on crypt depth ($F_{1,32}$=97.909, P<0.001 and $F_{3,32}$=75.452, P<0.001, respectively) with a highly significant interaction between time and treatment ($F_{3,32}$=41.908, P<0.001). While crypt depth remained comparable between day 14 and 22 in naïve and CsA treated groups of hamsters, a slight increase was observed in groups of hamsters with the primary infection on day 14 and this was considerably more marked by day 22.

6.3.3 – Cellular division in the crypt of Lieberkuhn

Cellular division in the crypts of Lieberkuhn was quantified. The mean number of mitotic figures observed in the crypts of hamsters given the four different treatments is illustrated in Figure 6.3. Analysis by two-way ANOVA revealed that there were no significant differences with time ($F_{1,32}$=0.183, P=0.672). In contrast, a highly significant main effect of treatment ($F_{3,32}$=33.477, P<0.001) and an interaction between time and treatment ($F_{3,32}$=7.999, P<0.001) were observed. With the mean of 4.3 figures/crypt ± 0.3 on day 14 and 4.2 mitotic/crypt ± 0.5, there were no sign of changes in the figures between the two days of the non-infected and non-treated group. On the other hand, mitotic figures in the crypts of all the remaining groups were elevated on both days (14 and 22) compared to the naïve control hamsters. CsA treated hamsters showed a reduction in the mitotic activities by day 22 (6 mitotic/crypt ± 1.9) compared to

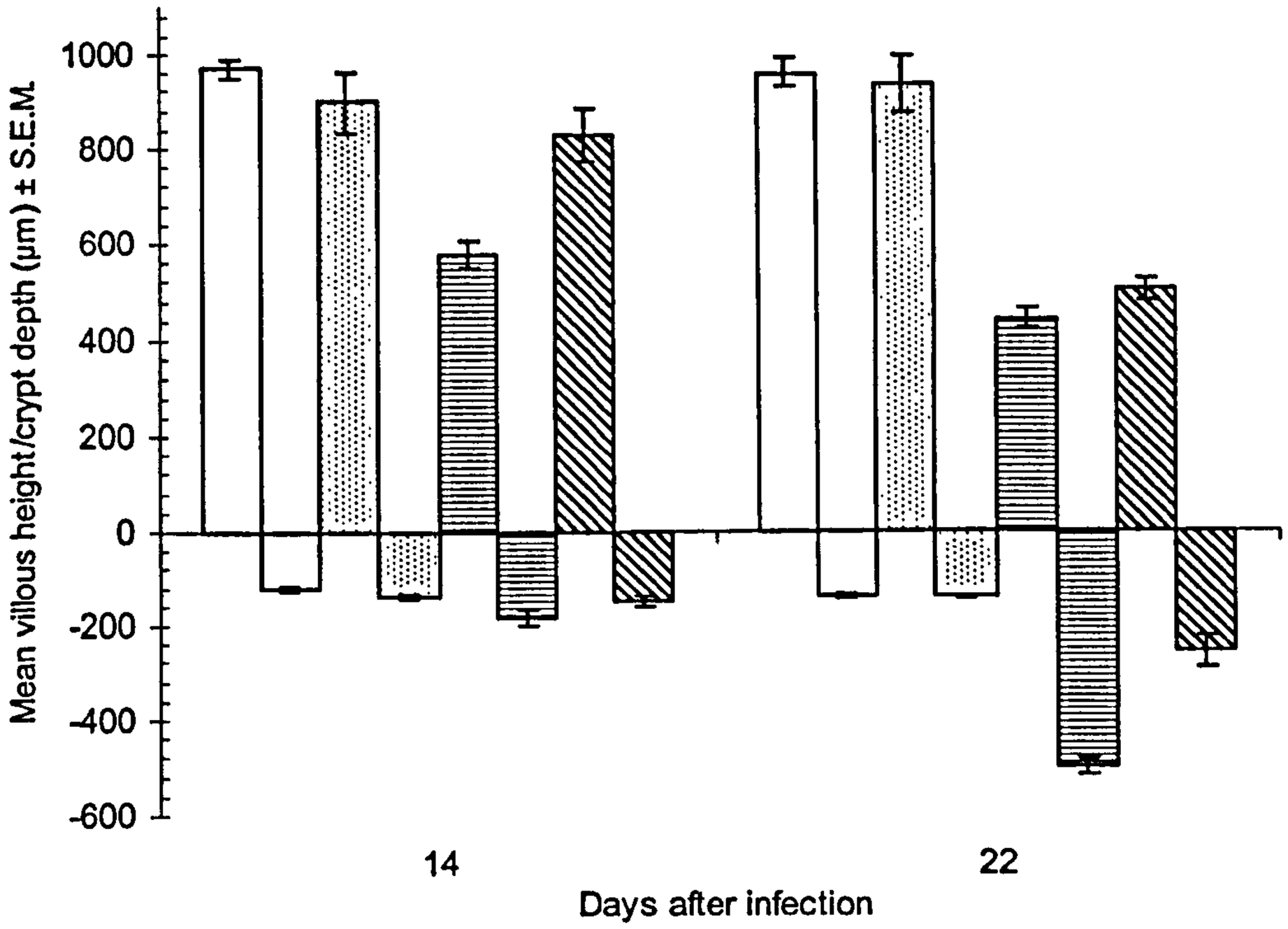


Figure 6.2 – Mean villous height+/crypt depth- (±S.E.M.) measured in the intestine of:

Naïve hamsters ( )

CsA-treated hamsters ( )

Primary infected hamsters ( )

Primary infected-CsA treated hamsters ( )

Statistical Analysis:

Analysis of these data by 2-way ANOVA with treatment (4 level, naïve, CsA treated hamsters, primary infected and primary with CsA treatment) and time (2 levels, 14 and 22) revealed:

Effect of treatment on villi ($F_{3,32}$=52.750, P<0.001), crypt ($F_{3,32}$=75.452, P<0.001)

Effect of time on villi ($F_{1,32}$=13.580, P<0.01), crypt ($F_{1,32}$=97.909, P<0.001)

Interaction Time x Treat. ($F_{3,32}$=7.536, P<0.01), crypt ($F_{3,32}$=41.908, P<0.001)

Model R^2 = 0.828 (villi) Model R^2 = 0.919 (crypts)

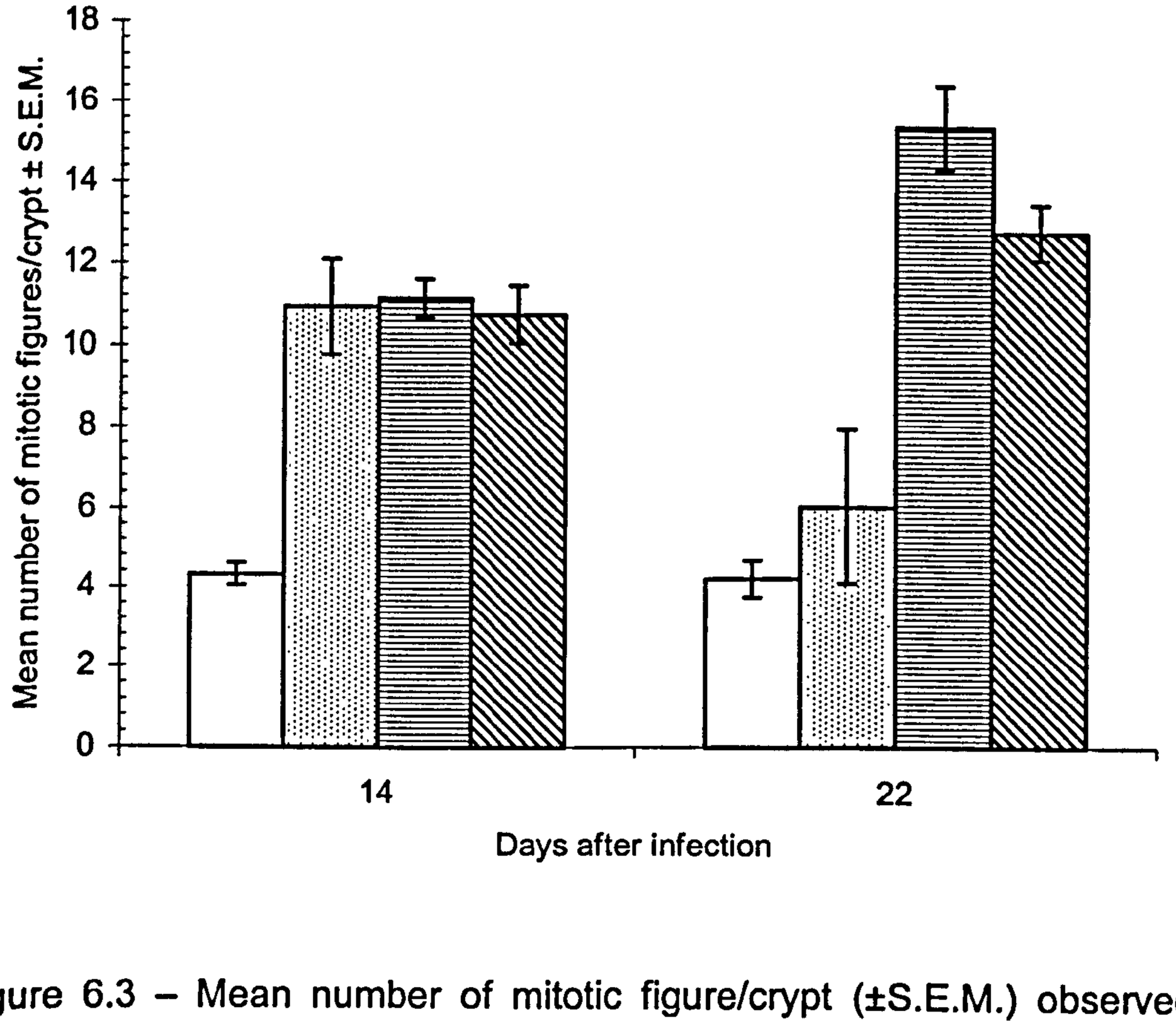


Figure 6.3 – Mean number of mitotic figure/crypt (±S.E.M.) observed in the intestinal crypts of:

Naïve hamsters ( )

CsA-treated hamsters ( )

Primary infected hamsters ( )

Primary infected-CsA treated hamsters ( )

Statistical Analysis:

Analysis of these data by 2-way ANOVA with treatment (4 level, naïve, CsA treated hamsters, primary infected and primary with CsA treatment) and time (2 levels, 14 and 22) revealed:

Main effect of treatment	($F_{3,32}$=33.477, P<0.001)
Main effect of time	($F_{1,32}$=0.183, P=0.672)
Interaction between treatment and time	($F_{3,32}$=7.999, P<0.001)

Model R^2 = 0.751

day 14 (10.9 mitotic/crypt ± 1.2). Both infected-nontreated, and infected CsA treated hamsters showed an increase in the number of mitotic figures observed between day 14 and day 22. The mean number of mitotic figure in the infected-nontreated group increased from 11.2 ± 0.5 on day to 15.4 ± 1.0 on day 22, but that in the infected and CsA treated group was elevated marginally (10.8 ± 0.7 on day 14 and 12.8 ± 0.7 on day 22).

6.3.4 – Mast cell responses

Figure 6.4 shows the mean number of mast cells in the intestines of the hamsters. There were no significant differences between treatments on day 14 and cells ranged between 9-14 cells/mm². However, by day 22, infected hamsters (with CsA treatment/ without CsA treatment) showed an increased in the mean number of cells. Mast cells rose to means of 27.5 ± 4.6 and 28.4 ± 3.3, respectively. Statistical analysis by two-way ANOVA gave the main effect of time and treatment ($F_{1,31}$=18.162, P<0.001 and $F_{3,31}$=15.284, P<0.001, respectively) as both significant. Moreover, a significant interaction between treatment and time ($F_{3,31}$=9.115, P<0.001) was also detected. Naïve hamsters and not-infected CsA treated hamsters showed no major differences in mast cells between day 14 and 22 whereas in infected hamsters, mast cell count rose to 13.8 ± 1.4 cell/mm^2 on day 14 and 27.5 ± 4.6 on day 22. There was little difference between this group and the infected and treated group.

6.3.5 – Goblet cell responses

Alcian blue – PAS stained cells were observed and counted to assess the number of goblet cells/mm² of intestinal tissue of hamsters (Figure 6.5). Goblet cell counts remained as low as 56.1 cells/mm² ± 4.9 on day 14 and 65.5 cells/mm² ± 3.1 on day 22 in naïve-noninfected hamsters with indistinguishable differences in goblet cell numbers between days 14 and 22. However, the numbers were slightly lower in groups of noninfected-treated hamsters and

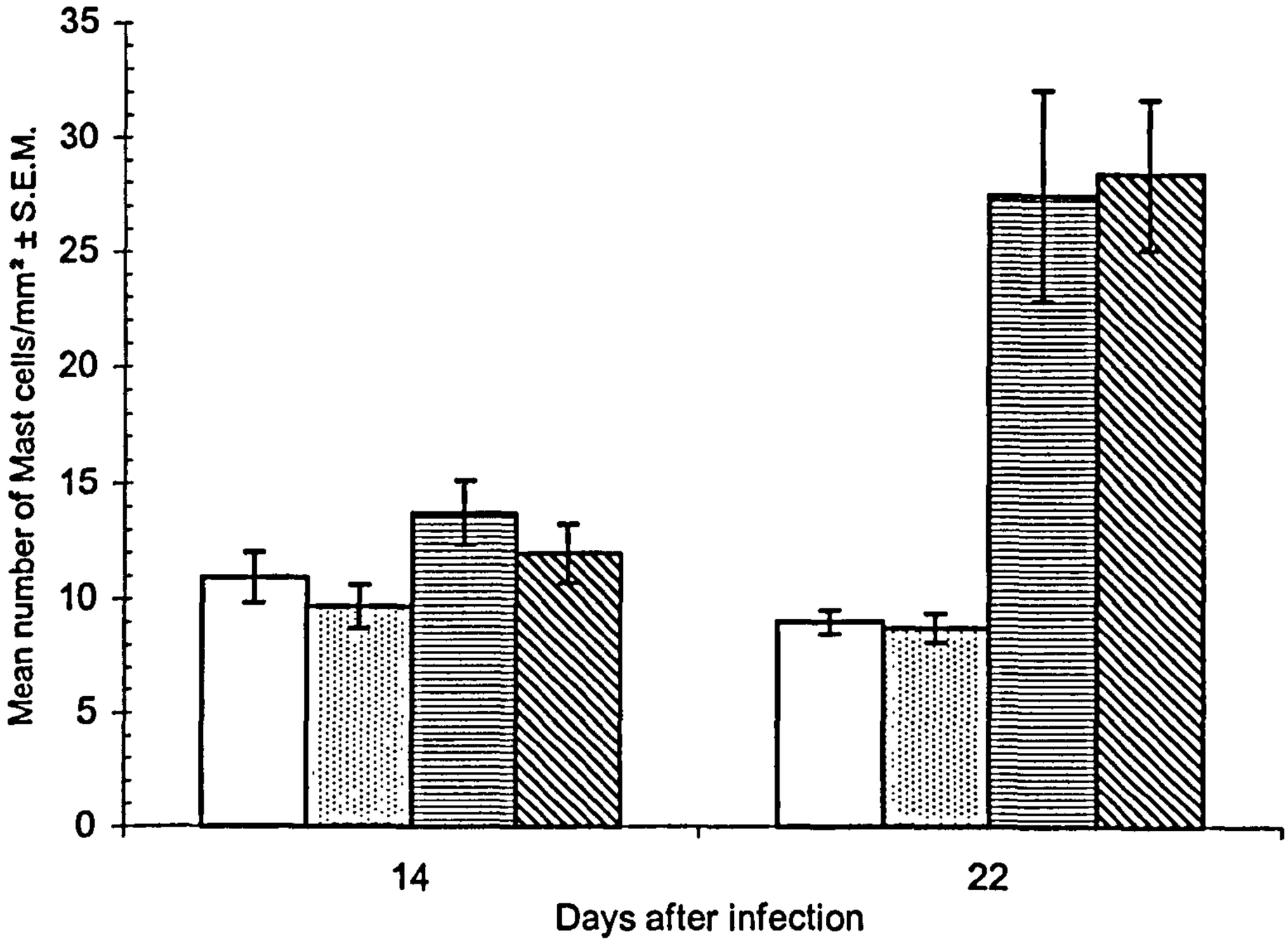


Figure 6.4 – Mean number of mast cells/mm² present in the intestinal tissue (±S.E.M.) of:

Naïve hamsters ( )

CsA-treated hamsters ( )

Primary infected hamsters ( )

Primary infected-CsA treated hamsters ( )

Statistical Analysis:

Analysis of these data by 2-way ANOVA with treatment (4 level, naïve, CsA treated hamsters, primary infected and primary with CsA treatment) and time (2 levels, 14 and 22) revealed:

Main effect of treatment	($F_{3,32}$=15.284, P<0.001)
Main effect of time	($F_{1,32}$=18.162, P<0.001)
Interaction between treatment and time	($F_{3,32}$=9.115, P<0.001)

Model R^2 = 0.692

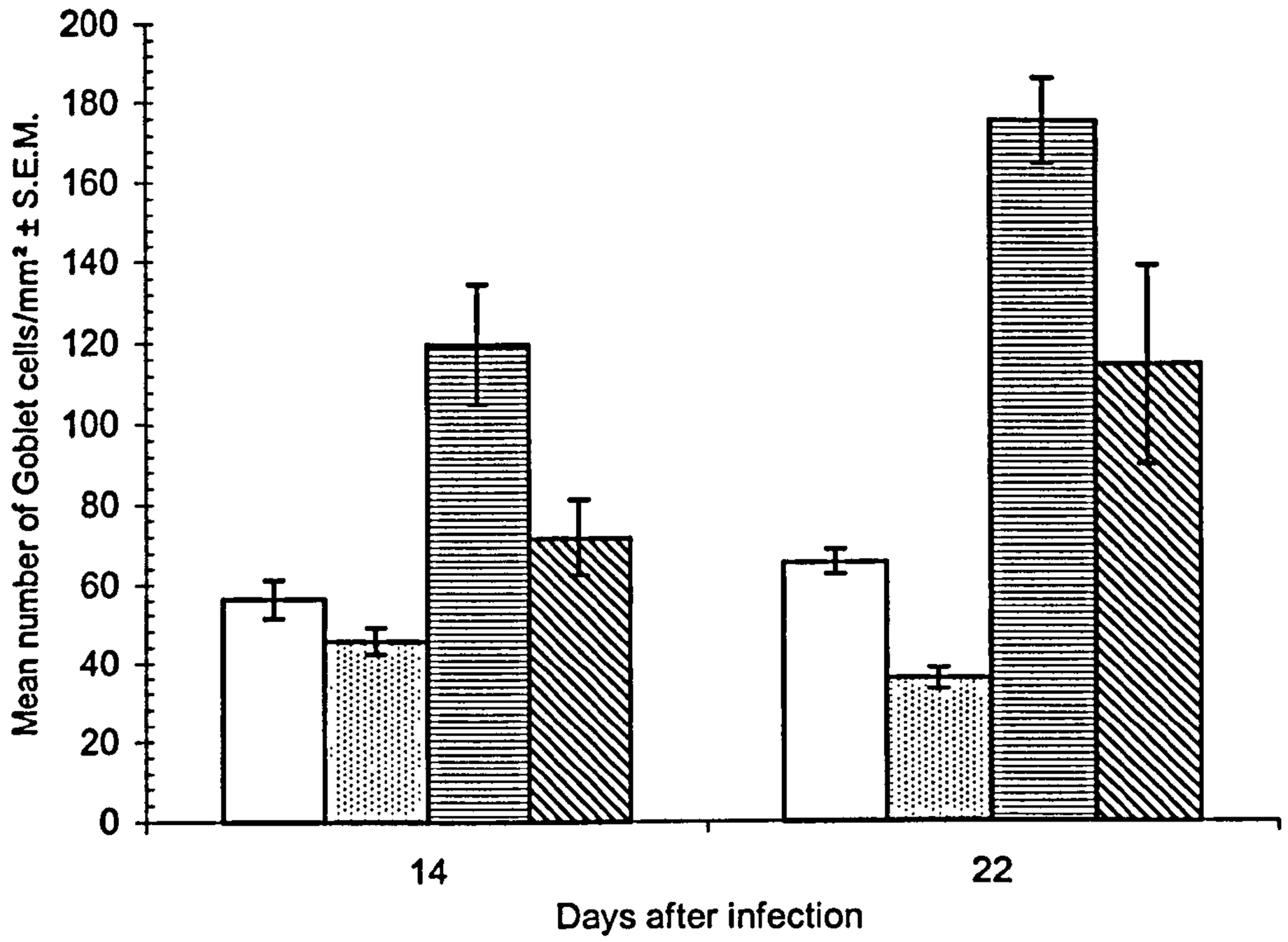


Figure 6.5 – Mean number of goblet cells/mm² present in the intestinal tissue (±S.E.M.) of:

Naïve hamsters ( )

CsA-treated hamsters ( )

Primary infected hamsters ( )

Primary infected-CsA treated hamsters ( )

Statistical Analysis:

Analysis of these data by 2-way ANOVA with treatment (4 level, naïve, CsA treated hamsters, primary infected and primary with CsA treatment) and time (2 levels, 14 and 22) revealed:

Main effect of treatment ($F_{3,31}$=40.201, P<0.001)

Main effect of time ($F_{1,31}$=10.672, P<0.01)

Interaction between treatment and time ($F_{3,31}$=4.068, P<0.05)

Model R^2 = 0.781

there was noticeable reduction in the same treatment from day 14 to 22. Goblet cell numbers in both infected groups of hamsters (with/without CsA treatment) increased relative to naïve-noninfected groups by day 14 and even more markedly by day 22. There was also a remarkable increase in goblet cells from 119.7 cells/mm² ± 14.8 on day 14 to 175 cells/mm² ± 10.7 on day 22 in the infected nontreated group. The graph shows a similar response, from 71.7 cells/mm² ± 9.4 on day 14 to 114.1 cells/mm² ± 24.6 on day 22 in infected treated hamsters. Two-way ANOVA analysis indicated that there was a significant main effect of time and treatment on goblet cells numbers ($F_{1,31}$=10.672, P<0.01 and $F_{3,31}$=40,201 P<0.001, respectively) and similarly, a significant main interaction between time and treatment($F_{3,31}$=4.068, P<0.05).

6.3.6 – Paneth cell responses

Changes in Paneth cells in the crypts of Lieberkuhn were counted. The mean number of cells is illustrated in Figure 6.6. As can be seen Paneth cell numbers were relatively higher in naïve noninfected groups (1.1 cells ± 0.1 on day 14 and 1.8 ± 0.2 on day 22) compared to all other treatments. However, a reduction was noted in groups, which received CsA only (0.9 ± 0.3 on day 14 and 1.2 ± 0.2 on day 22), and a more marked effect in hamsters that were infected (0.2 ± 0.03 on day 14 and 0.5 ± 0.2 on day 22). Analysis by two-way ANOVA indicated that time and treatments had significant effects on Paneth cells ($F_{1,32}$=21.759, P<0.001 and $F_{3,32}$=11.697, P<0.001, respectively) but there was no significant interaction between treatment and time ($F_{3,32}$=2.079, P=0.123).

6.3.7 – Eosinophil responses

The mean numbers of eosinophils counted in the intestines of hamsters are illustrated in Figure 6.7. The data show that there were no differences in the number of eosinophils between naïve and CsA treated hamsters with an

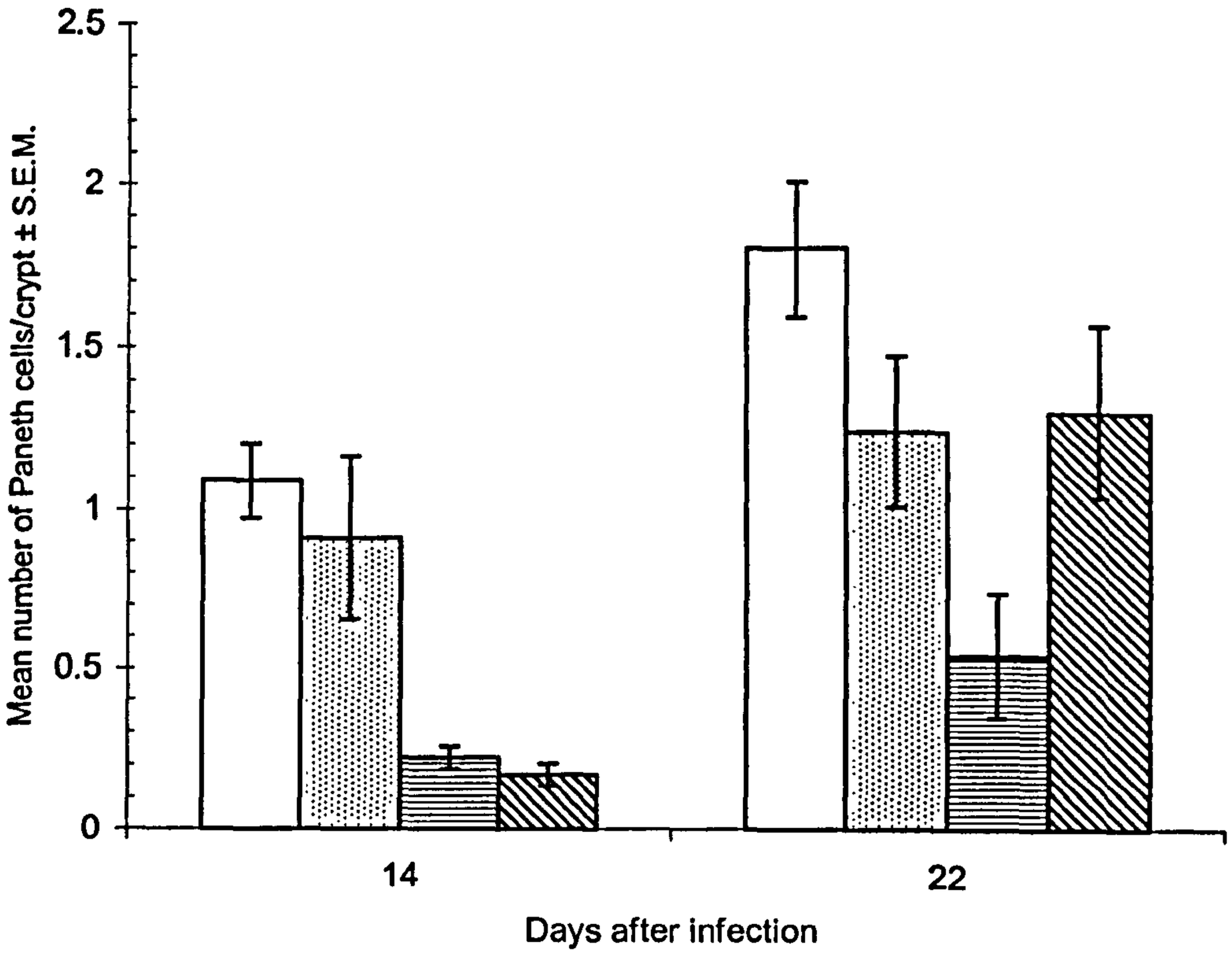


Figure 6.6 – Mean number of Paneth cells/crypt (±S.E.M.) present in the intestinal tissue of:

Naïve hamsters ( )

CsA-treated hamsters ( )

Primary infected hamsters ( )

Primary infected-CsA treated hamsters ( )

Statistical Analysis:

Analysis of these data by 2-way ANOVA with treatment (4 level, naïve, CsA treated hamsters, primary infected and primary with CsA treatment) and time (2 levels, 14 and 22) revealed:

Main effect of treatment ($F_{3,32}$=11.697, P<0.001)

Main effect of time ($F_{1,32}$=21.759, P<0.001)

Interaction between treatment and time ($F_{3,32}$=2.079, P=0.123)

Model R^2 = 0.590

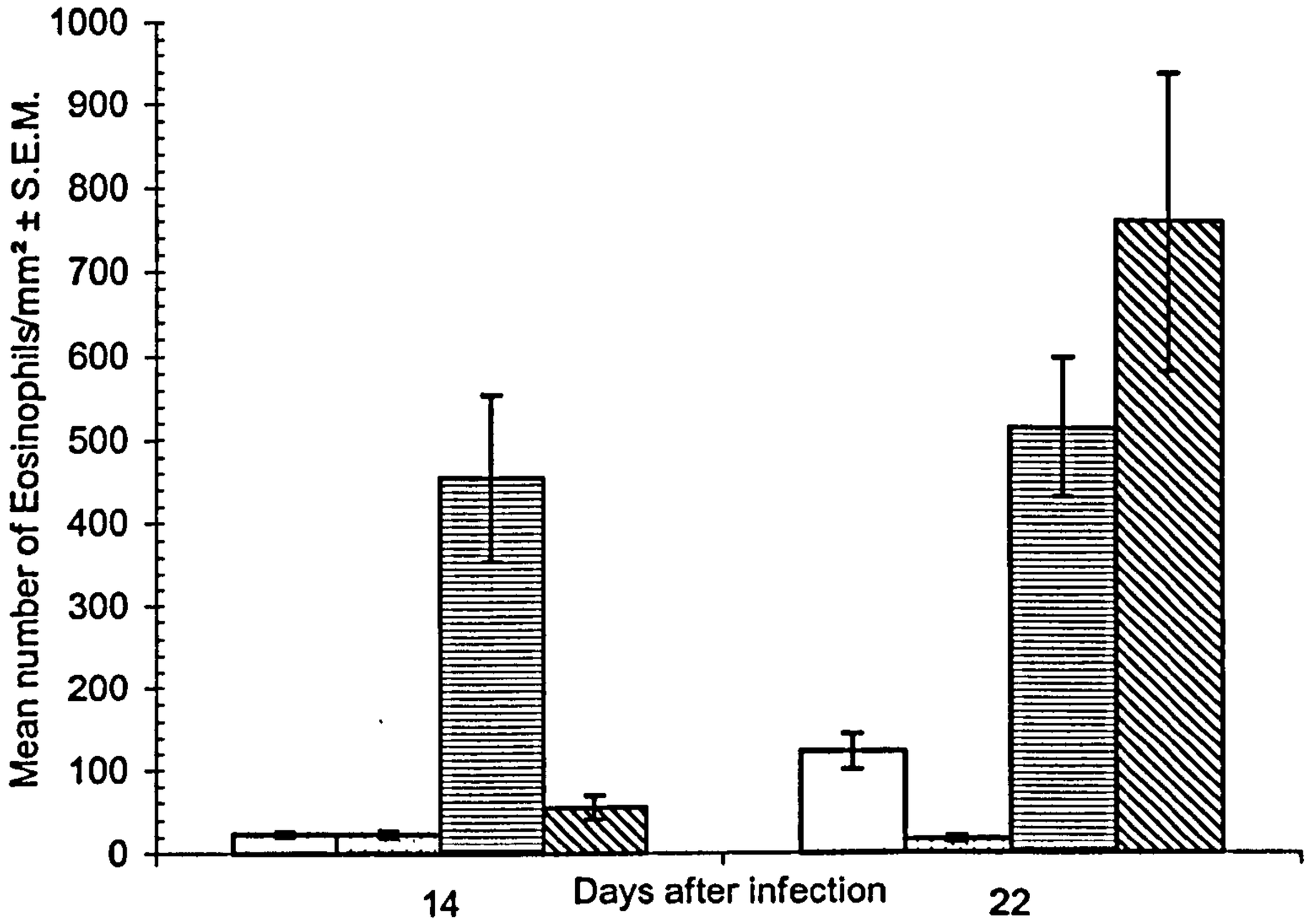


Figure 6.7 – Mean number of eosinophils/mm² present in the intestinal tissue (±S.E.M.) of:

Naïve hamsters ( )

CsA-treated hamsters ( )

Primary infected hamsters ( )

Primary infected-CsA treated hamsters ( )

Statistical Analysis:

Analysis of these data by 2-way ANOVA with treatment (4 level, naïve, CsA treated hamsters, primary infected and primary with CsA treatment) and time (2 levels, 14 and 22) revealed:

Main effect of treatment	($F_{3,31}$=22.679, P<0.001)
Main effect of time	($F_{1,31}$=18.712, P<0.001)
Interaction between treatment and time	($F_{3,31}$=10.519, P<0.001)

Model R^2 = 0.734

average of 16-25 cells/mm² on day 14. However, on day 22 there appeared to be a significant increase in noninfected and not-treated hamsters. However, a dramatic increase was noted in the infected-nontreated hamsters by days 14 and 22, with little variation between the two times. On the other hand, infected-treated hamsters showed differences between the two times (14 and 22). Analysis by two-way ANOVA revealed a highly significant main effect of time and treatment ($F_{1,31}$=18.712, P<0.001 and $F_{3,31}$=22.679, P<0.001, respectively) and a significant interaction between time and treatment ($F_{3,31}$=10.519, P<0.001).

6.4 – DISCUSSION

The experiment described in this chapter was designed to observe changes in the intestine of hamsters during hookworm infection while the host 's immune system was suppressed. Treatment of hamsters with CsA, at the dose levels used is known to induce suppression of the immune system in some murine models (Mclauchlan *et al.*, 1999; Oran *et al.*, 1997).

It is known that *A. ceylanicum* infections are generally stable during the first 7 weeks after infection (Garside and Behnke, 1989) and hence chronic. Prolonged treatment with CsA beyond week 3 could not be considered because of the possibility that immunosuppresion combined with hookworm infection would cause excessive pathology and suffering to the animals. Hence, this experiment was designed to observe the effect of treatment with CsA over a shorter periods of time, confined to the first 3 weeks, when marked changes in the intestine are already known to take place (Chapter 3 and 5). A careful record of hamsters' weight was maintained to ensure that animals did not suffer severely and to comply with the new Home Office regulations.

The data from this experiment shows that treatment with CsA resulted in a significant reduction in worm burden in hamsters infected with *A. ceylanicum* and treated with CsA, compared to those with infection only. The number of worms recovered from infected hamsters on day 14 was much as expected,

with worm burdens just less than 25 % of the total number of larvae administered, much as in Experiment 4 in Chapter 3 (Figure 3.9-B). These results also confirm the gradual loss of worms from control infected but non-treated animals between day 14 and 22, which was similar to that found in Chapter 5. The worm burdens recovered from infected-treated hamsters were markedly lower compared to the infected-nontreated and were very similar on both days indicating that no changes in burden had taken place after day 14. McLauchlan, *et al.* (2000) suggested that CsA damage the surface of parasites such as the cestode *R. microstoma* and it is possible that development stages of *A. ceylanicum* was also affected by damage to their surface, perhaps during the moult from L3 to L4 or L4 to preadult stages. The observation that worm burden did not change between 14 and 21 days post infection despite continual treatment of the host with CsA indicates that stages were no longer susceptible to the anthelmintic activity of the CsA. Whatever the stage of which *A. ceylanicum* were affected, the fact that worm burden were reduced in CsA treated hamsters means that interpretation of the mucosal changes in treated animals will be confounded by differences in worm burden among the groups infected with hookworms.

It would appear therefore that CsA does have wider ranging anthelmintic properties, such as those reported to cause a reduction of the development of cysticercoids (R. *nana*) from eggs in mice (Matsuzawa *et al.*, 1998) when used at the dosage level of 100 mg/kg. Khattab, *et al.* (1998) and Pons, *et al.* (1988) also confirmed that the anthelmintic properties of CsA in term of an antischistosomal effect on early stages of schistosomiasis. Other scientists indicated that the immature worms of *S. japonicum* are less sensitive to CsA *in vivo* than *S. mansoni,* which additionally displays marked age-dependent differences in its sensitivity to CsA (Caffrey *et al.*, 1999). Furthermore, studies on mice infected with *T. spiralis* revealed potent anti-parasitic effects (Bolas_Fernandez *et al.*, 1988). Such effects have been recorded in infections with the protozoans *Plasmodia* (Thommen_Scott, 1981), Leishmania (Behforovz *et al.*, 1986) and metazoa including filariae (Bout *et al.*, 1984). The accumulated evidence in schistosome infections suggests that the mechanism of the drug that damages the parasite is independent from the mechanism of its

suppression of host immunity (Serra *et al.*, 1999). The current results indicating an anthelmintic effect of CsA on hookworm are supported by studies on other parasites.

6.4.1 – Architecture changes in the intestinal mucosa

Changes in the intestinal architecture associated with parasitic and particularly *A. ceylanicum* infection are reported else where (Carroll *et al.*, 1984; Carroll *et al.*, 1985). Most research has been conducted on the intestinal changes associated with the presence of hookworms in the intestine in the absence of any immunosuppressive treatments (Carroll *et al.*, 1986)

Data from the current experiment again confirm the marked effect of hookworms on the architecture of the small intestine and the time-dependant, increase in both villous atrophy and crypt depth. Villous height of uninfected, non-treated hamsters remained relatively high ranging between 950-970 µm during the course of this experiment. This was consistent with earlier data presented in previous chapters (Chapter 3, 4 and 5) where villous height was approximately similar. Likewise, villous atrophy associated with *A. ceylanicum* primary infection on days 14 and 22 resembled the level of villous atrophy in experiment 2 and 3 of Chapter 3. The levels in those experiments were around 500 µm on day 14 and around 400 µm by three weeks after infection. Again, this was time dependant.

It is tempting to suggest that the CsA treatment, through the immunosuppressive properties of the drug, delayed the immune response and hence its effect on villi. However, the infected-treated animals had fewer worms and therefore the situation is complicated by this unexpected findings. It is not possible to dissociate this result, the contribution of lower worm burdens, from that of a potentially depressed immune system. It is reported in the discussion to Chapter 3 that villous atrophy may be due to more than one factor. On the other hand, there is damage resulting from the worms browsing on the mucosa and other accompanying immune responses. Experiments by Garside, *et al.*

(1992) using mice infected with *T. spiralis* provide evidences that when CsA is used, villous atrophy and crypt hyperplasia are prevented. Data presented earlier (Chapter 3, Experiment 5) established a dose-dependant effect on villous atrophy. Hence, if villous height depends only on the number of worms established in the intestine, then villous atrophy should be lower in infected-treated hamsters, which had lower worm burdens. Alternatively or additionally, villous atrophy is known to be dependent on the immune response, which is sustained as long as the worms are present. As indicated in Experiment 7 (Chapter 3), when worms were completely removed using anthelmintic treatment, villous height returned to normal levels within a few days.

The experiment also revealed associated changes in crypt depth in response to infection with *A. ceylanicum* and in response to treatment with the CsA. Crypts in the intestine of infected and non-treated hamsters were slightly deeper at two weeks compared to not-infected and not-treated animals and in agreement with the results from the experiments reported earlier (Chapter 3). However, interestingly, there was no sign of crypt hyperplasia in the infected-treated animals by day 14 in which the values for crypt depth were similar to those of not infected and not treated groups and these values were consistent with earlier findings. However, by day 22, infected-treated animals showed an increase in crypt depth but it was less than that in the infected and non-treated animals. Data from previous chapters showed that crypt hyperplasia is one of the characteristic features of hookworm infection and this is confirmed here by the increased in crypt depth slightly by day 14 and dramatically within three weeks. It was suggested in the discussion of Chapter 3 that the response in crypt depth is not worm burden dependent but rather it could be a T-cell mediated effect. If Crypt depth is not affected by CsA then we should observe some changes and a concurrent increase with villous atrophy as detected in Experiments 3 and 4 of Chapter 3. However, the much reduced response in crypt depth of infected and treated animals supporting the findings by Cummina *et al.* (1989) who stated that the CsA prevented intestinal crypt hyperplasia but did not affect villous length.

The mitotic activities in the crypts of non-infected treated animals were found to be interestingly different to those found during the primary infection

(Chapter 3). The average of mitotic figures was around 4 figures/crypt in the control naïve hamsters, which is comparable to findings in most of the earlier experiments. However, by week 2, the number increased to about 11 figures/crypt in groups of uninfected hamsters that received CsA treatment and were comparable to levels in other infected, infected and treated hamsters. It could be that the CsA had a direct effect on the cellular endothelial proliferation in the gut. However, this should be confirmed by further investigation in the future.

6.4.2 – Mast cell responses

The results of this experiment showed that the number of MMC in the intestinal mucosal tissue of not-infected, not treated hamsters was around 10cells/mm² of intestinal tissue and this was approximately the same average as that observed in earlier chapters (Chapter 3,4 and 5).

Infected and not treated animals showed a rise of MMC by day 14 pi compared to the number of MMC in the intestinal tissue of not-infected, not-treated hamsters and a clear increase with time to as much as 27 cells/mm² by the end of this experiment. Although the increase was consistent with other previous experiments reported in earlier chapters, here mastocytosis was slightly lower compared to Experiment 3 (with a primary infection) in Chapter 3 (Figure 3.14 -B) which might be attributable to the higher number of *A. ceylanicum* L3 larvae that established in hamsters in the latter case. It was found that mastocytosis was not affected by the subcutaneous injection of CsA in either treatment. This contrasts with reported studies in which the mast cell numbers in the intestinal mucosa are reported to decline after CsA treatment (Mclauchlan *et al.*, 1999; Oran *et al.*, 1997; Toyota *et al.*, 1996). However, data by Oran *et al.* (1997) showed that mast cell numbers are dose-dependently reduced in asebia mice, which is a model of chronic dermal inflammation, when CsA was administered. Thus, the unresponsiveness by mast cells to CsA treatment in this experiment could be related to the dose that had been used in this experiment. Data by Whalen *et al.* (1999) documented that there are

quantitative and qualitative species differences in both hepatic and gut metabolism of the CsA, measured by the disappearance of the CsA and the formation of the metabolites. This might support the previous idea that hamsters were less susceptible to CsA than other rodents. Their data was carried out on different species including humans, baboons, dogs, rabbits, hamsters and rats with results showing significant gut metabolism in dogs (~85%), hamsters (~78%) and rabbits (~68%) but not in rats (~6%) and gut metabolism was more variable compared with hepatic metabolism. Since the metabolism of CsA varies between species, this could be one explanation for the failure of CsA to suppress the mast cell response in the current experiment

6.4.3 – Goblet cell responses

Goblet cell numbers were similar to those quantified in previous experiments (Chapter 3,4 and 5) with the number of goblet cells remaining stable during the course of the experiment in naïve hamsters and never exceeding the mean of 70 cells/mm² of intestinal tissue. However, when hamsters were exposed to primary infection of *A.ceylanicum*, goblet cell numbers increased dramatically to the total mean number above 100 cells/mm² on day 14 and 3 folds higher by day 22. However, this finding contrasts with the results of the mean number of goblet cells on day 14 in Chapter 5 (Figure 5.6), where is no elevation in the mean number of goblet cells on day 14 when the mean numbers of worm established was around 16 worms. Nevertheless, the overall pattern of the goblet cell response on those days was similar to that reported in Experiment 3 of Chapter 3 (Figure 3.16 - B).

The suggestion, made in earlier chapters (Chapter 3, Experiment 5 and 6) that changes in goblet cells were mostly dependant on the dose of *A. ceylanicum*, could be supported by the results from this experiment. However, fewer worms were recovered in this experiment than those reported in Chapter 3, which might be due to the lower dose of larvae given, compared to those in earlier chapter. Since data in Experiment 3 (Chapter 3) and the experiment reported in Chapter 5 showed that the goblet cell response was dependant and

stimulated by adult worm numbers, the lower goblet cell numbers in infected hamsters treated with CsA, compared to those with a primary infection may reflect fewer worms in the former group.

6.4.4 – Paneth cell responses

The not-infected not-treated group of hamsters killed on day 14 had lower numbers of Paneth cells compared to those counted on day 22. The mean number of the total Paneth cell count was around 1 cells/crypt on day 14, reaching 2 cells/crypt in the later group. However, the number of Paneth cells in not-infected and not-treated groups contrasts with what was reported in Chapters 3 and 5 where the numbers ranged 3-6 cells/crypt. In this experiment, the number of Paneth cells in naïve hamsters was lower. However, all other groups showed fewer Paneth cells.

The pattern of the Paneth cell response to infection with *A. ceylanicum* was similar to that reported in the previous experiments of this thesis. However, contrasting data were found in Experiment 3 in Chapter 3 (Figure 3.16 - A) when the mean number of Paneth cells was above 1.5 cells/crypt on day 14 and about 1 cell/crypt by week 3 while here it is less than 0.5 cells/crypt on day 14. Nevertheless, the number of Paneth cells in this experiment was higher on day 22 and very close to that reported in Chapter 3 with wide variations between hamsters within groups .

Since the number of Paneth cells is quite different in this experiment between and within treatments, it would be interesting to explore the effect of adult worms only and larvae separately on the Paneth cell response and this could be done by giving L3 stages to hamsters then removing them by chemotherapy before maturation of worms. Adult worm could be given to hamsters by transplantation to observe the changes in Paneth cells due to adult worm infection alone.

6.4.5 – Eosinophil responses

The experiment reported in this chapter showed interesting findings regarding the infiltration of eosinophilia into the intestinal mucosa. Not-infected not-treated hamsters killed on day 14 showed very few eosinophils, with no more than 25 cells detected in mm^2 of intestinal tissue. This was in agreement with previous work presented in Chapter 5. However, this number was slightly increased on day 22 pi. It is suggested that this could have arisen as a result of enteric infection encountered in the animal house.

Interestingly, the eosinophil counts were markedly higher in infected, not-treated hamsters by day 14, but much lower in infected-treated animals. This suggests that CsA treatment prevented the eosinophil response in the first 2 weeks following infection. Data by Schopf, *et al.* (1998) from patients with psoriasis showed that CsA has an effect on eosinophils in dermal tissue. It has been found that treatment with CsA leads to a rapid drop of blood eosinophil counts. However, in the present experiment by day 22, the infected-treated hamsters had very high eosinophil counts, suggesting that there had been a marked but delayed response, and the effect of the CsA had worn off despite continued administration.

Finally, the experiment reported here was disappointing overall. It was carried out to test the hypothesis that the intestinal changes that were described earlier in this thesis were T cell driven. Because of the absence of appropriate methods for depleting T cell in hamsters and the absence of genetic mutants with T cell defects, the experiment was carried out using a drug known to have anti T cell effects. In other hosts, CsA is known to specifically impair T cell functions (Paavonen and Hayry, 1980; White *et al.*, 1979; Wiesinger and Borel, 1980). However, the present experiment suffered from 2 problems. Firstly, the haemagglutination assay, with which it had been hoped to demonstrate the immunodepressive properties of CsA, failed to generate a response to SRBC in naïve uninfected non-treated animals. So on this basis, it was not possible to conclude whether CsA had immunodepressive properties or not. Secondly, and unexpectedly, CsA turned out to have a significant anthelmintic effect so that

worm burdens were not comparable in the 2 infected groups. Some unexpected and odd trends, in term of the response in CsA treated animals, were identified earlier in this discussion but overall it is clear that this approach of using CsA to impair T cell function in hamsters was not successful. The formal demonstration that the cellular and architectural changes in the gut of hamsters infected with *A. ceylanicum* are driven by a Th2 response must therefore await confirmation through exploitation of other methodologies. Perhaps the easiest will be to raise a specific anti hamster T cells serum in rabbits and to use this to deplete T cells in hamsters (Xia *et al.*, 1999).

CHAPTER SEVEN

General discussion

7.1 – GENERAL DISCUSSION

Over 20% of the world's population has hookworm infection with a total estimated figure of over 900 million people infected worldwide (Chan, 1997; Pawlowski *et al.*, 1991). Hookworms are prevalent throughout the tropics and subtropics, wherever there is faecal contamination of the environment, and therefore the disease constitutes a considerable burden on the developing world (Gilles, 1985). The control of hookworm disease is theoretically simple and attainable through health education from childhood onwards. However, the improvement in hygiene necessary to avoid transmission seems unattainable in the short to mid-term (Cairncross, 1990). Although drug treatment is available, it is not always used and there is evidence of emerging drug resistance (Barnes *et al.*, 1995; Carroll and Grove, 1985b; Coles, 1998; Coles *et al.*, 1994; De_Clercq *et al.*, 1997; Geerts and Gryseels, 2000; Georgiev, 1999; Grover *et al.*, 2001; Jackson and Coop, 2000; Sacko *et al.*, 1999; Savioli *et al.*, 1997).

An alternative to chemotherapy is vaccination, however, a vaccine for human hookworm diseases is still not available. An effective vaccine for hookworm disease would have enormous benefits, particularly if it was able to induce a long period of protection. For this reason, there is growing interest in developing a vaccine for hookworm disease (Cypess and Zidian, 1975; Davis, 1987; Sen *et al.*, 2000; Vinayak *et al.*, 1981). The existing evidence for the feasibility of developing vaccines is based on more than 70 years of successful protection in laboratory animals with either antigens extracted from parasites or with attenuated larvae (Hotez *et al.*, 2003a; Hotez *et al.*, 2003b). Successful vaccination of BALB/c mice against human hookworm infection was demonstrated using gamma-irradiated *N. americanus* larvae (300 L3) (Girod *et al.*, 2003). Moreover, it was found by Ali, *et al,* (2001) that targeted vaccination, with recombinant subunit material derived from a known effective immuno-suppressant secreted by the parasite, may offer partial protection against the transmission of the hookworm *A. ceylanicum*. Whilst progress has been made with experimental immunizing protocols, relatively little attention has been given to understanding the pathology of hookworm infection, especially at the

intestinal stage. In order to facilitate the development of a vaccine, a deeper understanding of the host-parasite relationship is required, especially regarding aspects such as host immune responses and pathology attributable to both the parasite and the host response. Therefore, this thesis reports experiments that investigated intestinal mucosal changes in hamsters during infection with *A. ceylanicum*, a laboratory model system with many similarities to human hookworm infection.

Previous attempts were made to establish hookworms of various species, such as those of dogs and humans, in laboratory hosts such as mice and rats but such models showed no evidence of any chronic or patent infection being established (Chandler, 1935; Ray *et al.*, 1975). Mice infected with *A. ceylanicum* gave equivocal results in the attempt to produce patent infections. Experiments carried out by Ray, *et al.* (1975) on albino mice infected with *A. ceylanicum* larvae derived from a hamster adapted strain resulted in some fecund adult worms. Nevertheless, the system was difficult to interpret because the animals were immunosuppressed. Moreover, the attempts by Carroll, *et al.* (1983) to create patent infections in several strains of immunocompromised mice did not result in successful patent infections. A preliminary experiment in mice infected with *A. ceylanicum*, which was carried out by Garside (1998) and referred to in this thesis earlier, also produced no patency. Eventually, Ray, *et al.* (1972a, 1972b) infected hamsters with *A. ceylanicum* and their observations revealed successful adaptation of this canine species to hamsters. The parasite completed its development and produced fertile eggs from day 13 but usually not until day 17 (Ray *et al.*, 1972a). Since then, work based on hamsters infected with hookworms and particularly the canine species, *A. ceylanicum,* has been continued by many scientists (Brailsford and Behnke, 1992a; Garside *et al.*, 1989; Gupta and Katiyar, 1985; Gupta *et al.*, 1992). Research experiments have showed that the hookworm *A. ceylanicum* is capable of living in DSN hamsters for periods of between 5 to 7 weeks and up to 245 days (Behnke, 1990; Visen *et al.*, 1984). A more recent study showed that transferring adult worms from some species, such as Syrian golden hamsters or LVG(SYR) BR outbred strain (Charles River Laboratories) to Syrian hamsters resulted in long term survival and that adult worms survived for at least 42 days

after adult worm transfer (AWT) (Bungiro *et al.*, 2003). It is possible therefore to say that adult hookworms cause chronic disease even if the host is not exposed to larval stages.

In this thesis, I have investigated some aspects of the hamster's immune response to hookworm infection focussing mainly on changes in the intestinal architecture and the accompanying cellular responses in the mucosa of the intestine. This is a much neglected aspect of hookworm infections because of the ethical issues and difficulties associated with obtaining biopsy specimens from humans, and the impracticability of conducting large scale experiments in dogs. Thus most currently available information on mucosal changes during hookworm infection is based on case reports of human infections (Ottesen, 1990) and small numbers of dogs killed at different time during infection (Carroll, 1990). The work described in this thesis provides the first quantitative analysis of mucosal changes occurring in animals experiencing infection with hookworm, and exposed to a variety of infection protocols ranging from low dose single pulse through trickle exposure, challenge infection to immunosuppression.

The preliminary experiment reported at the beginning of the third chapter (Experiment 1 –Chapter 3) was carried out in order to investigate the effect of *A. ceylanicum* on the cellular mucosa in the intestine of hamsters. Two species of nematodes were used. The main species was the hookworm *A. ceylanicum* and the other species was *T. spiralis*. The later was included in order to provide comparative data for the magnitude of the response in hamsters, because the relationships between the host and the parasite *T. spiralis* are better documented having been investigated extensively (Alizadah and Murrell, 1984; Bell and Liu, 1988; Featherston *et al.*, 1992). The chronicity of *A. ceylanicum* hookworm infection was confirmed, as shown previously by other scientists (Behnke *et al.*, 1994; Bungiro *et al.*, 2003; Carroll and Grove, 1984).

It has been shown earlier that infection with nematode parasites produces marked changes in the intestine (Dehlawi and Wakelin, 2002; Ferguson and Jarrett, 1975; Ferguson *et al.*, 1980; Lawrence *et al.*, 1998; Mahida, 2003). Several experiments were carried out to determine the effect of *A. ceylanicum*

on intestinal architecture and the associated cellular changes in the intestine during primary infection. *A. ceylanicum* and *T. spiralis*, both caused cellular and architectural changes in the intestinal mucosa characterised by increasing depth of the crypts and reduction of the height of the villi. The results presented in Figure 3.5-A (Chapter 3) showed that villous height fell from the normal levels, which were usually between 900-1000 µm in naïve-uninfected hamsters to heights below 850 µm in hamsters infected with *A. ceylanicum* and 700 µm in hamsters infected with *T. spiralis* just 10 days after infection. Data from Experiments 3, 4 (Figure 3.10) and Experiment 7 (Figure 3.30) showed that the reduction in villous height continued with time and this was associated with a concurrent increase in crypt depth and mitotic figures in the crypt. These results generally are consistent with finding of Ferguson and Jarrett (1975) on rats infected with *N. brasiliensis*, work by Lawrence *et al.* (1998) on BALB/c mice infected with *T. spiralis* and Kamal (2001) on mice infected with *T. spiralis* that marked changes occur in the intestinal morphology during helminth infections. It was established here that as long as adult *A. ceylanicum* were present in the intestine, reduction in the height of villi together with an increase in the crypt depth occured. The mean values are changed significantly from those in uninfected animals despite variation in the mean number of worms between experiments (see Figure 3.9 and Figure 3.29). This could be due to several factors including worm feeding activities and probably immune responses targeting the site of infection by the host. The unchanged intestinal architecture in the early stages of infection (before day 10) and the reasonably quick return to normal value following anthelmintic treatment in Experiment 7 (Figure 3.30) provide evidence that adult worms are most likely to be the direct cause of these changes.

Mast and goblet cells are other parameters of the intestinal response that were quantified during hookworm infection. They have long been thought to be involved in the expulsion of different species of parasites (reviewed in Rothwell, 1989). Recently, Maruyoma *et al*, (2000) found that mast cells contribute to the expulsion of *S. venezuelensis* from mice by preventing the invasion of host intestinal mucosa by adults. They found that treatment with glycosaminoglycans, such as heparin; dextron sulphate or ChS-E, which are

similar to mast cells content, inhibited the binding of *S. venezuelensis* adhesion molecules to mucosal epithelium and thus the invasion of the gut mucosa. It would appear that while mast cells degranulated during infection with *A. ceylanicum*, the released product are affecting the adhesion of the hookworm to the intestine surface. However, the exact role of mast cells product needs to be investigated in relation with worm expulsion. Goblet cells also known to be involved in the expulsion of some helminths as they exclude and trap the nematodes living in the intestine by the secretion of the mucus. This forms a lubricant layer on the internal surface of the intestine, which reduces the ability of worm attachment to intestine. Both cells are believed to work together and/or individually to expel worms from intestine. Elevation in the number of mast cells and goblet cells has been reported often during GI nematode infections (Alizadah and Murrell, 1984; Alizadeh and Wakelin, 1982; Douch *et al.*, 1986; Kamal *et al.*, 2001; Loukas and Prociv, 2001; McDermott *et al.*, 2003; Miller and Nawa, 1979a). The experiment reported in Chapter 3 indicated that mast and goblet cells increased dramatically during the course of infection with *A. ceylanicum*. They also increased as the number of *A. ceylanicum* increased and therefore, these responses are both dependent on time and dose of parasites present in the intestine.

Although goblet cell counts were higher in hamsters with *A. ceylanicum*, they were even higher in animals with *T. spiralis* infection. The latter had higher parasite burden and this could have been simply a quantitative effects. However, other explanations are also conceivable and it may be that there are important antigenic differences between these two parasites that underlie the different responses. Different species of parasitic nematodes are known to differ antigenically and antigenic differences have even been detected within species between individual worms and between the sexes in *T. trichiura* (Currie *et al.*, 1998). This has implication for the development of immunity to parasitic nematodes. Accordingly, as such antigenic differences have been observed within one species of nematode, it is possible that antigens differ between individuals in other species. One additional difference is in the niche occupied in the intestine. *T. spiralis* penetrate enterocytes and create mucosal tunnels within which they live (Smyth, 1994). In contrast, hookworms are essentially

lumen dwellers that feed off the mucosal surface. These differences may also explain the varying patterns of response, but a comprehensive explanation must await further experimentation.

As with goblet cells, mast cell numbers increased dramatically in hamsters infected with *A. ceylanicum.* Mast cells have long been thought to be involved in the expulsion of different species of parasites (reviewed in Rothwell, 1989). Elevation in the number of mast cells has been reported often during GI nematode infections (Alizadah and Murrell, 1984; Alizadeh and Wakelin, 1982; Douch *et al.*, 1986; Kamal *et al.*, 2001; Loukas and Prociv, 2001; McDermott *et al.*, 2003; Miller and Nawa, 1979a). Figure 3.6 (Experiment 1) showed that mast cell counts were much greater in *A. ceylanicum* infected compared to *T. spiralis* infection, but both were greater than those in naïve hamsters. Hence, It would appear that mast cell responses may be more sensitive to hookworm infections in comparison with *T. spiralis* infections. This could be related to hookworm feeding activities involving the parasite embedding its anterior end in the mucosa to reach the blood circulation and therefore, the antigens, which are released by the parasites, make direct contact with the immune components in the circulation. It could also be attributable to differences in antigens between the species (*A. ceylanicum* and *T. spiralis*).

The results of mast cell counts in Experiment 3 in Chapter 3 (figure 3.14 (A) and goblet cells in figure 3.16 (A) showed fluctuations in the response and this could be attributable to inaccuracies of the conventional method of counting where the villi and crypts are damaged due to infection and thus cannot be easily viewed and counted. Therefore, the new Weible methods was used in Experiment 4 and this showed more consistent estimation of cells in the intestine, the data confirming that mast and goblet cell responses are time dependant.

The most interesting finding from this experiment was that the mast cell response took longer to return to levels typical of normal uninfected animals, whereas goblet cell numbers dropped back to normal levels once the adult worms had been removed with chemotherapy. Experiments with DSN hamsters infected with *T. spiralis* showed similar mast cell persistence after the loss of the

majority of adult worms (Behnke *et al.*, 1994). This suggests that mast cell and goblet cell responses are to some extent independently controlled in the intestinal mucosa. In mice, they are known to be regulated by different combination of Th2 cytokines. When Th2 cells are generated in the mesenteric lymph node and stimulated by a specific antigen, cytokine secretion begins and this includes the secretion of IL-3, IL-4, IL-9 and IL-10, which are required for mastocytosis. On the other hand, IL-13 is necessary for stimulation of mucus production (Else and Finkelman, 1999; Else and Grencis, 1991a; Finkelmen *et al.*, 1997; Nawa *et al.*, 1994). It could be that the secretion of these cytokines varied following removal of worms, or that the half-life of the mast cells and goblet cells differed such that goblet cells, being shorter lived, declined faster than mast cells. Support for this idea comes from the finding that goblet cells are actually produced from precursors within the mucosa and controlled by local factors including T-cells (Miller, 1984; Paulus *et al.*, 1993). As a result, changes in the gut may be more quickly responded to by goblet cells than mast cells, which have a slower turnover and more slow tendency to decline when the source of their stimulus has been removed (Garside *et al.*, 1990).

My experiments also investigated Paneth cells during hookworm infection. It was interesting to find that Paneth cells in hamsters responded differently to nematode infections than those seen in mice. The number of Paneth cells fell during *A. ceylanicum* infection in hamsters compared to that observed in naïve-uninfected animals (Experiment 2, Chapter 3) whereas data from Kamal (2001) showed that Paneth cells increased in the mouse intestine during *T. spiralis* infection. Satoh, *et al.* (1990) reported different types of Paneth cells in various mammals. He also found that the number and the size of secretory granules were also diverse among various animal species including humans, monkeys, hares, guinea pigs, bats, mouse, rat and hamsters. Secretory granules containing homogeneous electron-dense materials were observed in the Paneth cells of the first five species, whereas in the mouse the granules were bipartite. In hamsters, Paneth cells granules showed various electron densities (Satoh *et al.*, 1990). It was therefore pertinent to compare the response of Paneth cells between mice and hamsters infected with two species of nematodes. This was carried out in Chapter 3 (Experiment 2). The precise role of Paneth cells during

nematode infections still need to be investigated. Since hamsters also responded to *T. spiralis* infection by a reduction in Paneth cell numbers, it was concluded that the Paneth cell response differed from one host to another and that the species of the parasite may play a lesser role in this different pattern of response.

Eosinophils were also investigated during primary infection with *A. ceylanicum*. However, quantification of eosinophils in the intestinal tissue was introduced later in the course of the project and they were quantified only in Experiment 6. The results showed a significant and dramatic increase in the number of eosinophils in hamsters' intestines when infected with *A. ceylanicum* and this response seemed to be dose-dependant. The huge increase in their number agrees with other studies which showed that eosinophils play an important role in nematode infections (Behm and Ovington, 2000; Meeusen and Balic, 2000; Pritchard, 1995b; Scepek *et al.*, 1994; Stevenson *et al.*, 1994; Vardhani, 2003). The mechanism of eosinophil mediating protection against helminth infections still not fully understood. However, some studies showed that the mechanism could involve antibody-induced release; complement-induced release or toxic granule protein and reactive oxygen intermediated by activated eosinophils.

My experiments also investigated changes that occur during secondary infections with *A. ceylanicum* and two such experiments were carried out. The major finding was that the number of worms established in the intestine of hamsters after the abbreviated primary and then challenge infection was significantly lower than those established in control naïve hamsters receiving the challenge infection alone. This result confirmed previous finding by Behnke, *et al,* (1997) who revealed that previously infected animals acquired long-lived resistance to weight loss and anaemia reflecting the reduced capacity of adult worms to establish in the intestine of previously infected animals. Other authors have also found that *A ceylanicum* are affected during secondary infection when hamsters were either immunised with soluble adult hookworm antigens or following the removal of worms from the 1st infection (Bungiro *et al.*, 2001). However, here the parasite burden was higher in hamsters when the second infection of *A. ceylanicum* (Experiment 2, Chapter 4) was superimposed.

Therefore, resistance to the challenge infection was more marked in the absence of adult worms persisting from the primary infection. This suggests that persisting adult worms, in the primary-superimposed challenge infection groups (Chapter 4, Experiment 2) facilitated the establishment of challenge infection larvae, possibly by down regulation of some components of immunity. Hookworm have been suggested to secrete immunomodulatory molecules (Ball and Bartlett, 1969; Barriga, 1984; Pritchard *et al.*, 1988) and despite the intense mucosal response as reflected in mastocytosis and goblet cell hyperplasia, it may be that some of the underlying components necessary for worm expulsion were depressed.

Villous height and crypt depth during abbreviated primary-challenge infection with *A. ceylanicum* showed similar responses to those seen during primary infection reported in Chapter 3. The villous height was nearly at normal levels then declined continuously with time to levels close to those recorded during primary infection. Crypt depth also increased with time, from normal levels but exceeded the level observed in hamsters with primary infection alone. However, the mitotic figures showed no signs of any change during the first two weeks of the challenge infection but then eventually increased significantly to more than 20 figures/ crypt, which is double the number counted in primary infected hamster. This suggests therefore that the villi are affected by the direct number of worms present in the intestine whereas crypt depth and mitotic activities may also reflect to some extent protective immune mechanisms of the host.

Infiltration by some inflammatory cells (e.g. mast cells) was shown to differ between abbreviated primary-challenged groups and primary-superimposed challenge infection hamsters. Experiment 1 (Chapter 4) reported a slight increase from day 73 to 94 (7 to 28 days after challenge) in the number of mast cells in the groups of abbreviated primary-challenged hamsters. It would appear that the number of mast cells fell following the removal of the primary infection and remained at levels similar to those seen in naïve-not challenged groups. Then from day 35, one week after the secondary infection, mast cell numbers begun to increase and yet already at this time much of the secondary infection had been rejected or failed to establish. A suggestion from this finding is that

mast cells declined following the removal of the initial primary infection but their contents, which would have been released during the primary immunizing infection into the blood stream, may still have remained in the blood of hamsters and possibly even until after challenge. Another suggestion for the slow increase in mast cell numbers following the second exposure is that since the worm burden did not establish very well, the slow increase in mast cells after the first week of the secondary infection might be due to larvae from the challenge dose that actually managed to establish, developing, perhaps slowly as reported in Chapter 3.

The response of mast cells in the abbreviated primary – challenged groups in Experiment 2 (Chapter 4) was slightly different because the number of mast cells was already elevated, then fell by two weeks after challenge and then increased by the end of the experiment. The only suggestion to explain this is that the number of mast cells did not drop back to the normal levels after treatment with Ivermectin because some adult worms persisted from the immunized infection and therefore mast cell numbers were higher. When they started to fall, developing larvae from the new infection that had managed to establish began to stimulate the mast cell response.

Goblet cell responses were also observed following secondary infection including in both abbreviated primary-challenged and primary-superimposed challenge infections. The number in the first regime increased from a normal low level to a peak above 200 cells/mm^2 by week two of the challenge infection then fell down to less than 150 cells/mm^2. This response peaking at the end of week 2 following challenge infection again suggests that those larvae from the challenge infection that developed to pre-adult and adult stage provided triggers for goblet cell proliferation and when the number of worms fell slightly in the last week (Figure4.1, Chapter 4), the number of goblet cells dropped also (see Figure 4.5, Chapter 4).

Changes in the number of Paneth cells were also quantified. The number of Paneth cells in abbreviated primary-challenged and superimposed animals of Experiment 2 (Chapter 4) indicated no real difference between the two infected

groups. However, the number was very close to that found in normal uninfected hamsters and rather higher in Experiment 1 (Chapter 4).

My experiments also investigated the effect of trickle infections on the cellular response in the hamster's intestine. The number of worms established in the primary infected hamsters was greater than that found in the trickle infected groups. However, generally, both worm burdens declined with time. It was interesting to find that the worm burdens from trickle infections in Chapter 5 were very low compared to those of the primary infection. The major finding is that changes in the mucosal architecture and the associated cellular response were affected significantly by the incoming larvae. Infected groups of hamsters showed a decline in villous height, increase in the crypt depth and mitotic activities compared to naïve-uninfected hamsters. They also showed that mast, goblet and eosinophils increased with time but not Paneth cells, which declined with time compared to uninfected animals. However, these responses differed between the trickle and primary infection and this could be due to the higher number of adult worms established in the intestine of hamsters with primary infection. Similarly, The results of this trickle infection experiment suggest that resistance is induced very rapidly and that apart from the larvae derived from the first few inocula, few others manage to establish. These findings accord with those reported by Brailsford and Behnke (1992a).

The experiment in the last experimental chapter (Chapter 6) concluded that immunosuppression using CsA resulted in loss of worm from the intestine (Figure 6.1, Chapter 6). Hamsters given the primary infection showed a total of 20 worm in the intestine on day 14 post infection and around 10 one week later. In contrast, the number of worms never exceeded a total of 5 at both times in hamsters treated with CsA. CsA is known to be a powerful immunosuppressive drug, which inhibits T cell responses. Its property of acting as an anthelmintic has been reported previously in other animal models but not in hamsters nor during infection by hookworms (Bolas_Fernandez *et al.*, 1988; Hurd *et al.*, 1993; Kocken *et al.*, 1996).

Changes in the villi and crypts also differed as a result of treatment and infection. Infected and CsA treated hamsters showed longer villi and shorter

crypts than those in primary infected groups. This suggests that CsA may have depressed the immune mechanisms by binding to the intracellular receptor cyclophilin (CyP) to prevent cellular degranulation and the production of cytokines that normally drive changes in mucosal architecture. However, the confounding problem for our interpretation is that worm burden were also lower in CsA-treated animals, presumably as a consequence of the anthelmintic properties of CsA (Hiramine *et al.*, 1989). It is possible that some stages of *A. ceylanicum* are prevented to establish normally in the intestine by the CsA as the case of rodent infected with some species of tapeworms and reported by Chappell *et al*, (1989). Other possibility is that the dose of CsA which has been used during our experiment may resulted in worm were killed due to the lethal of dose-dependent effect of the CsA on some species. This anti-parasitic effect of CsA are believed to be mediated via cyclophilin (CyP) (Colebrook *et al.*, 2002). All other cell types also showed similar responses with numbers of cells in the primary infected groups being higher than those in animals that were infected then treated with CsA. In contrast, Paneth cells and eosinophils responded differently in groups of hamsters that received infection and were treated with CsA. They showed more marked changes by day 22 of infection even thought the number of worms was very similar between days 14 and 22. The number of Paneth cells returned to a level that is similar to that found in CsA-only treated hamsters, which was double that seen in infected hamsters. The number of eosinophils in infected and CsA treated hamsters reached the highest level on day 22 of the experiment in Chapter 6 compared with other treatments (Naïve, CsA-only treated and infected hamsters). Overall, these results suggest that the outcome of treatment with CsA depends on the anthelmintic effect rather than inhibition of the immune initiators. Therefore, another type of immunosuppressive treatment will have to be considered in any future work.

In summary it can be seen that the *A. ceylanicum* / hamster model offers good opportunities for investigating the immune responses that occur during hookworm infection. Experimental work in dogs is very costly and ethically undesirable. Similar data cannot be obtained readily from infected people, again because of ethical considerations. Therefore the hamster-*A. ceylanicum* model,

which is currently the only model available in rodents where hookworms mature and cause chronic infections, provides a unique opportunity to understand the immunological relationships involved. The interplay between the parasite's feeding activities on the mucosal surface and the immune responses that it elicits, in bringing about the architectural changes reported in this thesis, is not easy to unravel. Perhaps the most important question for future work will be to examine the consequences of treatment with an immunosuppressive agent that does not have anthelmintic activity.

The work described in this thesis concentrated on a model system and the data on mucosal responses is original and novel. Hopefully it will be possible to continue the work further, to eventually achieve a more comprehensive understanding of the events that occur in the mucosa during hookworm infection and how these relate to protective immunity, parasite feeding activities, and parasite survival strategies.

Work along these lines will be important if we are to understand how effective immune responses can be stimulated against hookworms, despite their very successful survival strategies (Pritchard, 1995a). Effective vaccines for hookworm diseases are urgently required to control the vast number of infections throughout the world. It is hoped that the work reported in this thesis has made a significant contribution to our understanding of mucosal immunity in hookworm infections and this in turn provides support for vaccine development. The combination of modern molecular technologies, the capacity to clone and express parasite antigens in bulk, and an understanding of immune responses in the tissues where parasites live, should all come together to provide the basis for the eventual successful development of a vaccine for hookworm disease. It is the authors' wish that the work described in this thesis makes a useful contribution to facilitating this ultimate achievement.

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APPENDIX

GENERAL SOLUTIONS:

(A) PHOSPHAT BUFFERED SALINE pH 7.4

NaCl	**8.0g**
KCl	**0.2g**
$Na_2HPO_4 12H_2O$	**1.15g**
KH_2PO_4	**0.2g**

PH adjusted to 7.4 and the final volume is 1 litre with distilled water

(B) Hanks' Saline

Solution 1

NaCl	**168.0g**
KCL	**8.0 g**
KH_2PO_4	**4.0 g**
0.2% Phenol red	**200ml**

Made up to 2 litres with distilled water

Solution 2

$CaCL2.2H_2O$	**3.92g**
$MgCL2.6H_2O$	**2.0 g**

Made up to 2 litres with distilled water.

110 ml of Solution 1 were added to 110 ml of solution 2 up to 1 litre with distilled water. The pH was then adjusted to 702 with 1ml of NaOH.

STAIN AND FIXATIVES:

(A) Carnoy's fixative

Absolute alcohol (Ethanol)	**60ml**
Chloroform	**30ml**
Glacial acetic acid	**10ml**

(B) 10 % Formaldehade solution – 38% w/V

Formaldehyde	**100cc**

Made up to 1 litre with distilled water as fixative

(C) Clark fixative

Absolute alcohol (Ethanol)	**75ml**
Glacial acetic acid	**25ml**

(D) MT fixative

This fixative contains two solutions

Solution 1

Mercuric Chloride	**5.0 g**
Distilled water	**95ml**

Solution 2

Tannic acid	**20g**
Distilled water	**80ml**

Equal volume from each solution is mixed up after filtration of solution 2 and can be used up to maximum of seven days.

(E) 4% paraformaldehyde

Paraformaldehyde	**4%**
Phosphate buffer	**0.2M**

Was adjusted at pH 7.2

(F) 10% OF 40% FORMALDEHYDE

Formaldehyde	**10% of 40%**
Phosphate buffer	**0.15ml**

Adjusted at pH 7.2

(G) KARNOVSKY'S

12% of 25% Glutaraldehyde

37% of 8% paraformaldehyde in 0.2 M phosphate buffer (pH 7.2)

12.5% of 0.2 M Phosphate buffer (pH 7.2)

38% distilled water

This fixative used in two different temperature (4°C and 60°C).

(H) 1% Alcian blue

Alcian Blue	**1.0g**
0.7N HCl	**100ml**

(I) ALCIAN BLUE (pH 2.5)

Alcian Blue	**1.0g (BDH)**
3% Acetic acid	**100ml**

(J) SEFRANIN (0) 0.5% pH 1.0

Safranin	**0.5g (Gurr)**
0.125N HCl	**100ml**

(K) Ehrlich's haematoxylin

Haematoxylin	**2.0 g**
Ethanol	**100ml**
Distilled water	**100ml**
Glycerine	**100ml**
Glacial acetic acid	**10ml**
Potash alum	**In excess**

Ripened for six months

(L) 1% PERIODIC ACID

Periodic acid	**1.0g**
Distilled water	**100ml**

(M) SCHIFFS REAGENT

Basic Fuchsin	**1.0g**
Distilled water	**200ml**

(N) Tartrazine

Tartrazine	**5.0g**

2-ethoxyethanol (cellosolve)	**100 ml**

(O) Differentiation solution

0.7N HCl

(P) Phloxine

Phloxine	**0.5g**
Calcium chloride	**0.5g**
Distilled water	**100ml**

(Q) Carazzi's haematoxylin

Haematoxylin	**1.5g**
Saturated aqueous potassium or ammonium alum	**700ml**
1% iodine in 95% alcohol	**50ml**
Distilled water	**250ml**

(R) Chromotrope 2R

Phenol	**1.0g**
Chromotrope powder	**0.5g**
Absolute Ethanol	**10ml**

Distilled water	**100ml**

Finally, mixed up and filtered.

(S) Digestion medium

Pepsin A (BDH)	**2.5g**
Concentrated HCl	**2.5ml**
Distilled water	**500ml**

Made up in a 1 litre plastic beaker and the temperature are kept at 37°C before and during the digestion. Meat is left for 2 hours. For faster digestion, pepsin and conc. HCl were doubled and were used in Experiment number 5 of dose-dependant.

(U) Agar 0.2%

Agar	**20g**
Distilled water	**100ml**

(T) Erythrocyte lysis medium

Ammonium chloride (BDH)	**4.25g**
Distilled water	**500ml**

Adjusted to pH 7.2 with 1M NaOH filter sterilised.